1964

# Studies on the mechanism of action of the bioflavonoid Hesperidin methyl chalcone in causing vascular and intestinal smooth muscle to relax

Alan Brooks Combs
*University of the Pacific*

# STUDIES ON THE MECHANISM OF ACTION OF THE BIOFLAVONOID HESPERIDIN METHYL CHALCONE IN CAUSING VASCULAR AND INTESTINAL SMOOTH MUSCLE TO RELAX

---

A Thesis

Presented to

the Faculty of the Department of Physiology-Pharmacology

University of the Pacific

---

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

---

by

Alan Brooks Combs

June 1964

This thesis, written and submitted by

Alan Brooks Combs ,

is approved for recommendation to the

Graduate Council.

Department Chairman or Dean:

Carl C. Riedesel

Carl C. Riedesel

Thesis Committee:

Carl C. Riedesel, Chairman

James R. Thompson Charles A. Matuszak

Charles H. Koncz D. Conrade Head

Dated May 15 - 1964

ACKNOWLEDGEMENTS

The author wishes to express appreciation to Professor Carl C. Riedesel for guidance and encouragement throughout this study. Thanks are also due to Sunkist Growers for the grant that made this study possible.

TABLE OF CONTENTS

# LIST OF TABLES

# LIST OF FIGURES

FIGURE PAGE

# CHAPTER I

## INTRODUCTION

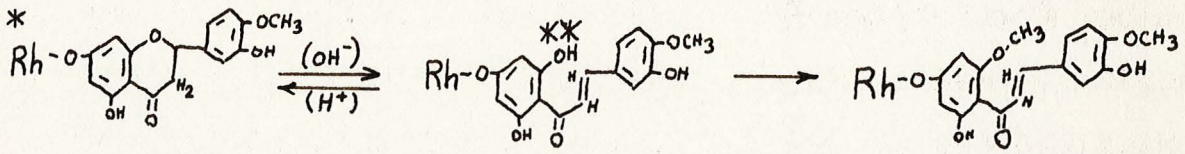
This thesis constitutes a report on a series of studies undertaken on possible mechanisms of action of the bioflavonoid, hesperidin methyl chalcone (HMC), in causing smooth muscle to relax. The term bioflavonoid refers to several compounds that can be extracted from the mesocarp of citrus fruits. They have been the subject of investigation and controversy since 1936. Much of this is covered in a review by Vogin (1). As mentioned in the review, Szent-Gyorgyi observed first that crude citrus extracts were more efficient in relieving experimental scurvy than pure extracts containing only Vitamin C. The subject of scurvy has been covered in many reviews, and no purpose would be served in describing all these factors at this time. However, since Szent-Gyorgyi's observation, there has been considerable study of the possibility that bioflavonoids are a factor in capillary integrity. Despite all the activity, the necessity of bioflavonoids as a dietary adjuvant has not been established. At this date, almost thirty years after the initial studies, the literature is replete with contradictory statements on almost every phase of bioflavonoid activity.

In spite of the somewhat extensive literature dealing with these compounds, there are few references as to the

acute effects following parenteral administration. Kikuchi, et al (2), reported that in anesthetized dogs, cats, and rabbits methylhesperidin caused a relatively slow fall in blood pressure as a result of reduced vascular tone. They reported that bioflavonoids had little effect on the rate and contractility of isolated perfused rabbit heart, while the coronary vasodilating effect was about one-fourth that of theophylline. Aramaki, et al (3), found that methyl-hesperidin, of uncertain chemical composition, at a dosage level of 50 to 100 milligrams per kilogram caused an obvious depression of blood pressure in rabbits and cats in proportion to the dose given. It also inhibited the rate and contracting power of rabbit atrium; this effect was reversible by washing. In an unpublished series of tests, Riedesel (4) observed that certain bioflavonoid compounds when given by intravenous injection to anesthetized cats had specific acute hypotensive effects ranging from mild to severe and prolonged.

In view of the limited studies on blood pressure reduction resulting from bioflavonoid administration, an effect not explained in the literature, it was decided to investigate the action of one compound, HMC, in order to elucidate the mechanism or mechanisms involved. HMC was selected for study for several reasons: (a) its availability as a reasonably pure compound, and (b) a ready solubility facilitating intravenous administration. This compound is

prepared by a semisynthetic process from the naturally occuring bioflavonoid, hesperidin. Hesperidin is insoluble in water but can be converted to soluble hesperidin chalcone by alkali as illustrated in Figure 1. As seen in the illustration, appropriate methylation prevents acidic reconversion of the chalcone to hesperidin, resulting in a permanently soluble form, hesperidin methyl chalcone. The molecular weight of the commercial compound varies from 640 to 670, depending on the degree of methylation (5).




(Hesperidin) (Hesperidin Chalcone) (Hesperidin Methyl Chalcone)

Figure 1. Interconversion between hesperidin and hesperidin chalcone.

*Rh refers to the sugar rhamnose.

**This phenolic group is one of those methylated in the formation of HMC, the methylation of which prevents reconversion to hesperidin.

# CHAPTER II

## METHODS AND PROCEDURES

### I. MATERIALS

All chemical reagents and drugs used throughout this investigation are listed in the Appendix.

### II. EXPERIMENTAL

The possibility that HMC might have a direct action on smooth muscle indicated that both *in vivo* and *in vitro* studies should be utilized. The *in vivo* studies consisted of observations on blood pressure and nictitating membranes of cats. The *in vitro* studies were performed on sections of rabbit ileum.

#### In Vivo Studies

The animals used in these studies were common domestic cats obtained from a licensed supplier of biological materials. They were of both sexes and were maintained on Purina Cat Chow and water, ad libitum. The animals were anesthetized with an intraperitoneal injection of sodium pentobarbital, 50 milligrams per kilogram. Blood pressure was directly recorded from the left femoral artery via a cannula to a blood pressure transducer. Impulses from the transducer were recorded on a Physiograph Six, a multichannel penwriting instrument manufactured by the E & M Instrument Company.

Norepinephrine and acetylcholine were injected prior to administration of HMC to standardize the response of the animal. Then they were injected after administration of HMC to determine if the bioflavonoid caused a departure from the previous responses. The possibility that HMC might modify vessel tone by a cholinergic mechanism was put to the test by ascertaining what changes, if any, atropine sulfate produced in the response to the bioflavonoid.

The relative accessibility of the superior cervical sympathetic ganglion of the cat made possible a series of nictitating membrane studies to determine what ganglioplegic or adrenolytic effects might result from administration of HMC.

In Vitro Studies

Preliminary work indicated that rabbit ileum was quite sensitive to the inhibitory effects of HMC. Because of the ease of recording the inherently rhythmical contractions, this type of smooth muscle was selected for the studies.

The rabbits, of the type known as California Giant (Oryctolagus species unknown), were obtained from a licensed supplier of biological materials. They were maintained on Purina Rabbit Chow and water, ad libitum. Both sexes were used. Each animal was fasted from eight to twelve hours prior to use. A typical ileum preparation was obtained in the following manner. A rabbit was stunned by a sharp blow to the back of the head and was immediately exsanguinated.

The abdomen was shaved with electric clippers and an incision was made along the linea alba. One and one-half to two centimeter sections of ileum were gently divested of mesentery. These sections were sutured at both ends and removed from the rabbit and were immediately placed in refrigerated Tyrode's solution (see Table I) at 3° C., for no longer than eight hours before use.

The method employed in the intestinal strip studies was modified after Hoff and Geddes (6). A muscle strip was allowed to contract in Tyrode's solution contained in a 20 milliliter capacity muscle warmer. The solutions were maintained at a temperature of 37.5° ± 0.5° C. Intermittent aeration was accomplished with a small respiratory pump. The muscle warmer was arranged to permit rapid drainage of one fluid and substitution of another. Contractions of the muscle strips were recorded by a Myograph B transducer on the Physiograph.

Changes in electrolyte composition. The minimum effective quantity of HMC required to inhibit muscle contraction in 20 milliliters of Tyrode's solution was determined empirically and found to be 10 milligrams. This concentration was then used as the standard concentration for all succeeding studies, including studies where the muscle strips were caused to contract in solutions using modified

TABLE I

STOCK SALINE SOLUTIONS PREPARED FOR SMOOTH MUSCLE STUDIES*

| Electrolyte | Standard Tyrode's Solution (A) | Ca++ Free Tyrode's Solution (B) | K+ Free Tyrode's Solution (C) | 8X K+ Tyrode's Solution |
|---|---|---|---|---|
| NaCl | 7.88 | 7.98 | 8.03 | 6.82 |
| KCl | 0.20 | 0.20 | -- | 1.60 |
| $CaCl_2 \cdot 2H_2O$ | 0.26 | -- | 0.26 | 0.26 |
| $MgCl_2 \cdot 2H_2O$ | 0.10 | 0.10 | 0.10 | 0.10 |
| $NaH_2PO_4 \cdot H_2O$ | 0.05 | 0.05 | 0.05 | 0.05 |
| $NaHCO_3$ | 1.00 | 1.00 | 1.00 | 1.00 |
| Dextrose | 1.00 | 1.00 | 1.00 | 1.00 |

The standard Tyrode's solution (A) was diluted with an appropriate quantity of modified solution (B or C) to prepare the modified ionic solutions shown in Table II.

*All values are expressed as grams per liter of solution.

TABLE II

A SUMMARY OF THE ION CONCENTRATIONS EMPLOYED IN EVALUATING THE EFFECT OF HMC ON SMOOTH MUSCLE CONTRACTION*

| Variations In K+ Ion Levels | Variations In Ca++ Ion Levels |
|---|---|
| 0.000 | 0.000 |
| 0.050 | 0.098 |
| 0.100 | 0.153 |
| 0.150 | 0.185 |
| 0.200** | 0.228 |
| 1.600 | 0.260** |

*All values are expressed in grams of potassium or calcium chloride per liter.

**Represents the amount of the ion found in standard Tyrode's solution.

concentration of electrolytes. The composition of stock solutions prepared for this work is shown in Table I. The differences in tonicity resulting from changes in concentration of an electrolyte were adjusted by appropriate modification of the sodium chloride concentration using the sodium chloride equivalent method (7).

Solutions of differing ionic composition were selected originally as a matter of convenience, but sufficient variations were introduced to provide a range from 0 to 100 per cent of the calcium or potassium in Tyrode's solution. In addition, a series of solutions was prepared with an eightfold increase in potassium ion as compared to standard Tyrode's solution. These experimental solutions were prepared just prior to use from the stock solutions and are listed in Table II. Intermediate solutions (e.g., one containing one-half or one-fourth the concentration of a certain ion in standard Tyrode's solution) were prepared by a dilution of Tyrode's solution with the particular stock solution without that ion.

In each experiment, the muscle was allowed to assume a constant height and rate of contraction in prewarmed Tyrode's solution. When the muscle was stabilized, there was a rapid replacement of the Tyrode's solution with the experimental solution; the time for complete exchange required less than ten seconds. To this experimental solution

was then added the HMC, a total of 10 milligrams dissolved in 0.5 milliliters of 0.85 per cent saline solution. Each experiment was repeated a minimum of eight times. In several instances, a maximum of twelve successive experiments were performed in order to assure reliability and reproducibility. The observed results were recorded as changes in height of contraction, plotted against time.

Cardiotonic glycoside studies. Cardiotonic glycosides are known to enhance contractility of cardiac muscle by altering the way in which the cellular membrane handles potassium (8). On the assumption that cardiotonic glycosides might act in a similar manner on intestinal smooth muscle, a limited number of experiments were conducted to determine if the glycosides from digitalis would modify the response to HMC.

Comparison of HMC with sodium versenate. Calcium chelating agents such as disodium ethylenediaminetetraacetate (sodium versenate) have been shown to inhibit contraction of glycerated skeletal muscle by Ebashi (9) and of vascular smooth muscle by Waugh (10). The probable explanation for the inhibition is that calcium requisite for contraction is rendered unavailable to the contractile proteins (9). The possibility that HMC might inhibit muscle by calcium chelation was explored. Empirical studies determined

that the minimum effective concentration of sodium versenate needed to inhibit smooth muscle was between 10 and 15 milligrams per 20 milliliters of Tyrode's solution. This amount of the chelating agent was added to muscles contracting in a solution with no potassium ion and the results were compared to those produced by HMC under the same conditions. In addition, the calcium chelating abilities of HMC and sodium versenate were compared. Two different soluble electrolytes which form insoluble calcium precipitates were added to calcium chloride solutions containing either water, HMC, or sodium versenate. The amount of precipitant needed to cause permanent turbidity of a solution depended upon the amount of unchelated calcium present. The solutions used are listed in Table III. The results were recorded as milliliters of titrant needed to cause permanent turbidity and are presented in the chapter on Results.

TABLE III

SOLUTIONS USED TO COMPARE THE CALCIUM BINDING ABILITIES OF APPROXIMATELY EQUIMOLAR SOLUTIONS OF HMC AND SODIUM VERSENATE*

| Experimental Solution | Trial 1.# (15 milliliters per solution, each containing 0.033% calcium chloride plus the substance indicated). | Trial 2.## (30 milliliters per solution, each containing 1.7% sodium potassium tartrate plus the substance indicated). |
|---|---|---|
| Water Controls | ---- | ---- |
| Solutions Containing Sodium Versenate | Sodium versenate (0.24%). Only two determinations. | Sodium versenate, (0.24%). |
| Solutions Containing HMC | HMC (0.34%). | HMC (0.34%). |

*Three determinations were made on each solution except where noted.

#In Trial 1, 0.1 per cent sodium oxalate was added to each solution from a burette until development of a permanent turbidity consisting of calcium oxalate.

##In Trial 2, 2.0 per cent calcium chloride was added to each solution from a burette until development of a permanent turbidity consisting of calcium tartrate.

# CHAPTER III

## RESULTS

### In Vivo Studies

The raw data for the in vivo studies are compiled in the Appendix. The blood pressure measurements are presented as per cent increases or decreases, with the pressure at the time of injection taken to be 100 per cent. This provides a useful means of comparing blood pressure effects of different dosages and agents when the initial pressure varies widely from animal to animal. Comparisons between challenge doses and doses given after HMC for several agents with vascular effects are presented in Table IV. Parts A and B of Figure 2 graphically present these comparisons for norepinephrine and acetylcholine.

Injection of atropine sulfate, 0.5 milligrams per kilogram, did not greatly change the response to HMC when given before or after the bioflavonoid. The atropine did abolish the blood pressure effects of intravenously administered acetylcholine. Parts C and D of Figure 2 demonstrate the differing effects of atropine on the vascular response to HMC and to acetylcholine.

Stimulation of the preganglionic fibers of the left superior cervical ganglion caused contractions of the left nictitating membrane and dilations of the pupil that were

TABLE IV

COMPARISON BETWEEN CHALLENGE DOSES
AND DOSES GIVEN AFTER HMC*

| Agent and Dose | Blood Pressure Responses (as per cent changes) Before HMC | After HMC | Remarks |
|---|---|---|---|
| Acetylcholine, 1.0 mcg./Kg. | 66 fall | 63 fall | No change. |
| Norepinephrine, 0.2 mcg./Kg. | 44 rise | 46 rise | The variations between the before and after responses are considered to be of no significance. |
| 0.2 mcg./ Kg. | 16 rise | 19 rise | |
| 0.2 mcg./Kg. | 14 rise | 25 rise | |
| 1.0 mcg./Kg. | 37.5 rise | 38 rise | |
| Epinephrine, 0.2 mcg./Kg. | 26 rise | 58 rise | Greater rise in pressure after HMC. |
| Pituitary Extract, 2.5 units | 41 rise | 27 rise | The vascular bed seemed less reactive after HMC. |

*Tabulated from three animals.

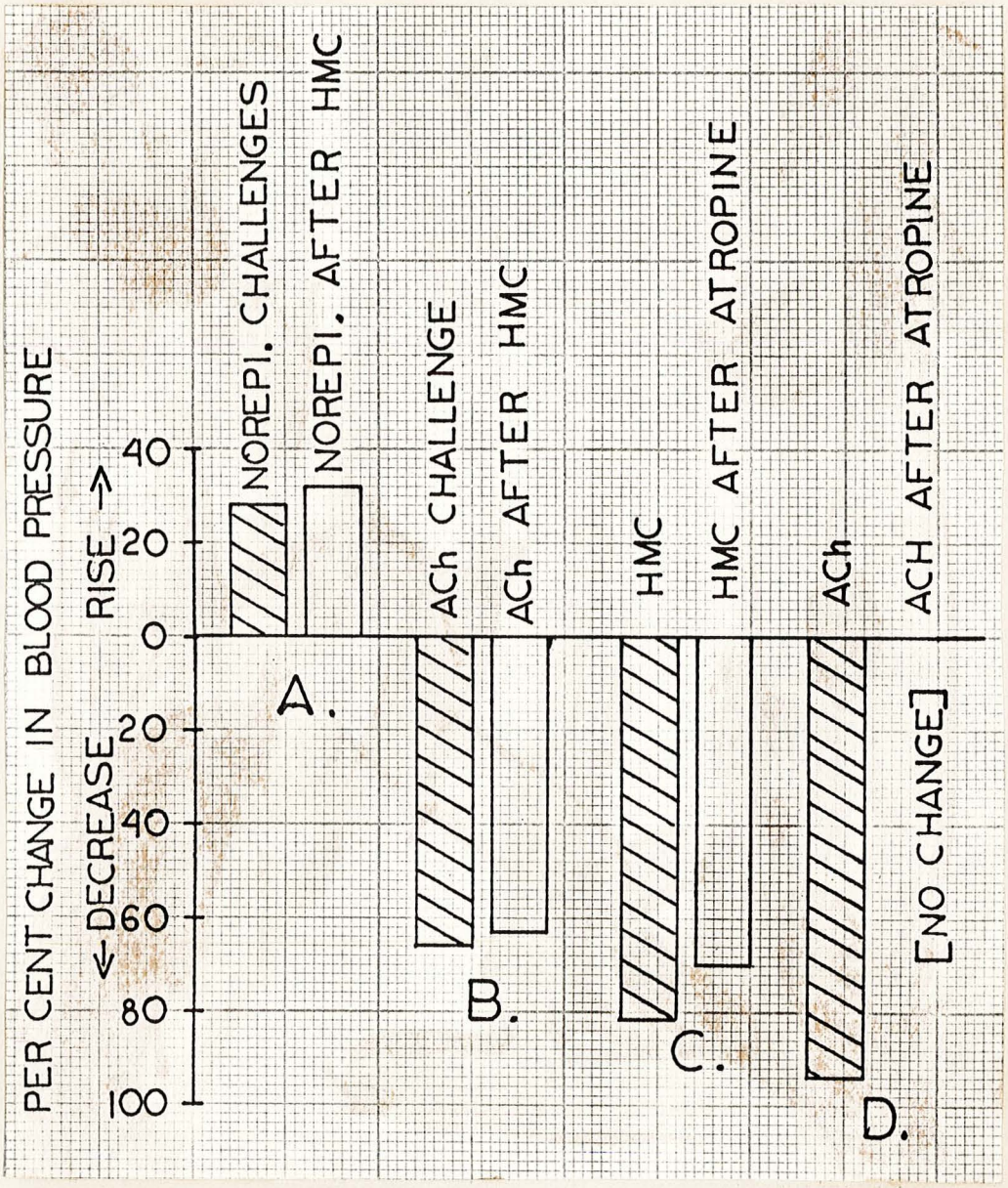



Figure 2. Representative blood pressure changes caused by norepinephrine, acetylcholine, and HMC.

A. Average increase caused by norepinephrine given before and after HMC (three animals).
B. Changes caused by acetylcholine, 1.0 mcg./Kg., when given as challenge dose and after HMC (one animal).
C. Changes caused by HMC, 50 mg./Kg., given before and after atropine sulfate, 0.5 mg./Kg.
D. Changes in pressure caused by acetylcholine, 2.0 mcg./Kg., given before and after the same dose of atropine given in C.

undiminished by large doses of HMC (up to 150 milligrams per kilogram). The response to preganglionic stimulation was greatly reduced by the administration of the adrenergic blocking agent phentolamine hydrochloride. The response was completely abolished in another animal by the ganglionic blocking agent hexamethonium chloride.

## In Vitro Studies

Changes in ionic composition. The experiments were performed as described in the chapter on Methods. The records were analyzed in the following manner. Actual contraction excursions of the recording pen were converted to percentages of initial contraction which allowed comparison of records with differing initial heights of contraction. The percentage values were plotted against time in seconds following exposure to HMC. The data from which the graphs were drawn appear in the Appendix. Because there was some variation among the contraction heights of the members of a series, a line representing the mean per cent height of contraction was plotted against time for each experimental condition. The mean was calculated at time intervals representing 3, 6, 12, 20, and 34 seconds after addition of HMC to the perfusing solution. Figure 3 illustrates the mean response to HMC of muscles contracting in Tyrode's solution. The mean height of contraction for muscles contracting in Tyrode's solution

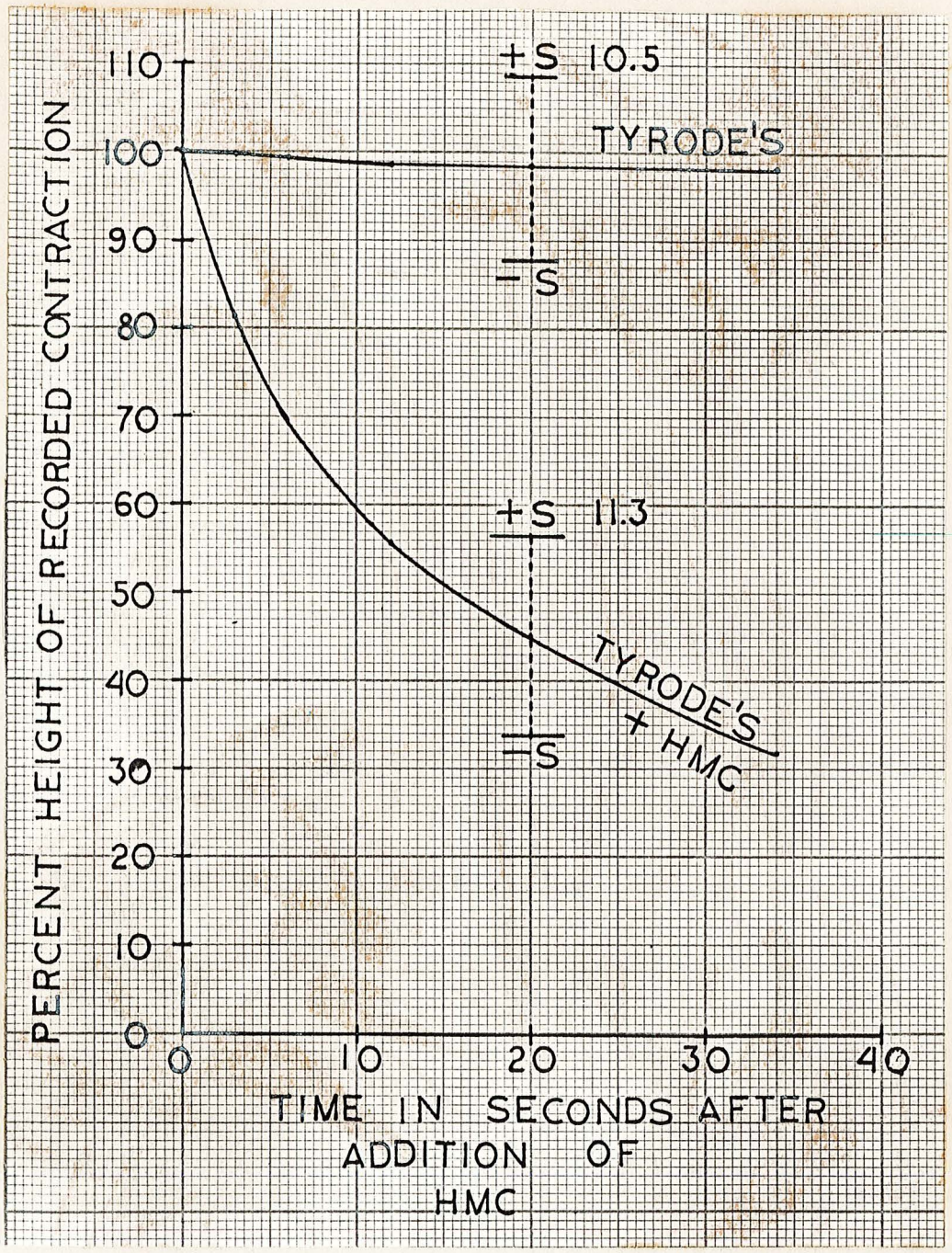



Figure 3. Comparison between mean heights of contraction for muscles contracting in Tyrode's solution (12 trials) and for muscles in Tyrode's solution to which HMC was added (12 trials). (The difference between the line at 20 seconds is significant at the 0.99 level.)

(when no HMC was added) is also shown in Figure 3 for comparison. Statistical analysis (Student-t) was performed on the two curves at the distance representing 20 seconds and the difference between the curves was found to be significant at the 0.99 level. The standard deviations in both cases are essentially the same and are probably representative of the variability inherent in this particular biological system.

Figures 4 through 12 show the responses to HMC obtained in the several ion deficient solutions. In each figure, the curve representing the response to HMC of muscles in Tyrode's solution is added for comparison and the level of statistical significance between the two curves is noted. The curves representing responses significantly differing from the response obtained in Tyrode's solution are compiled in Figure 13.

The effect of addition of 10 milligrams of HMC to muscles in Tyrode's solution (Figure 3) was a profound inhibition of contraction. The modified Tyrode's solutions that provided significant antagonism to the inhibitory action of HMC were those containing one-half or less of the potassium or calcium in standard Tyrode's solution.

A typical record, obtained when the Tyrode's solution was exchanged for a solution containing an eightfold increase

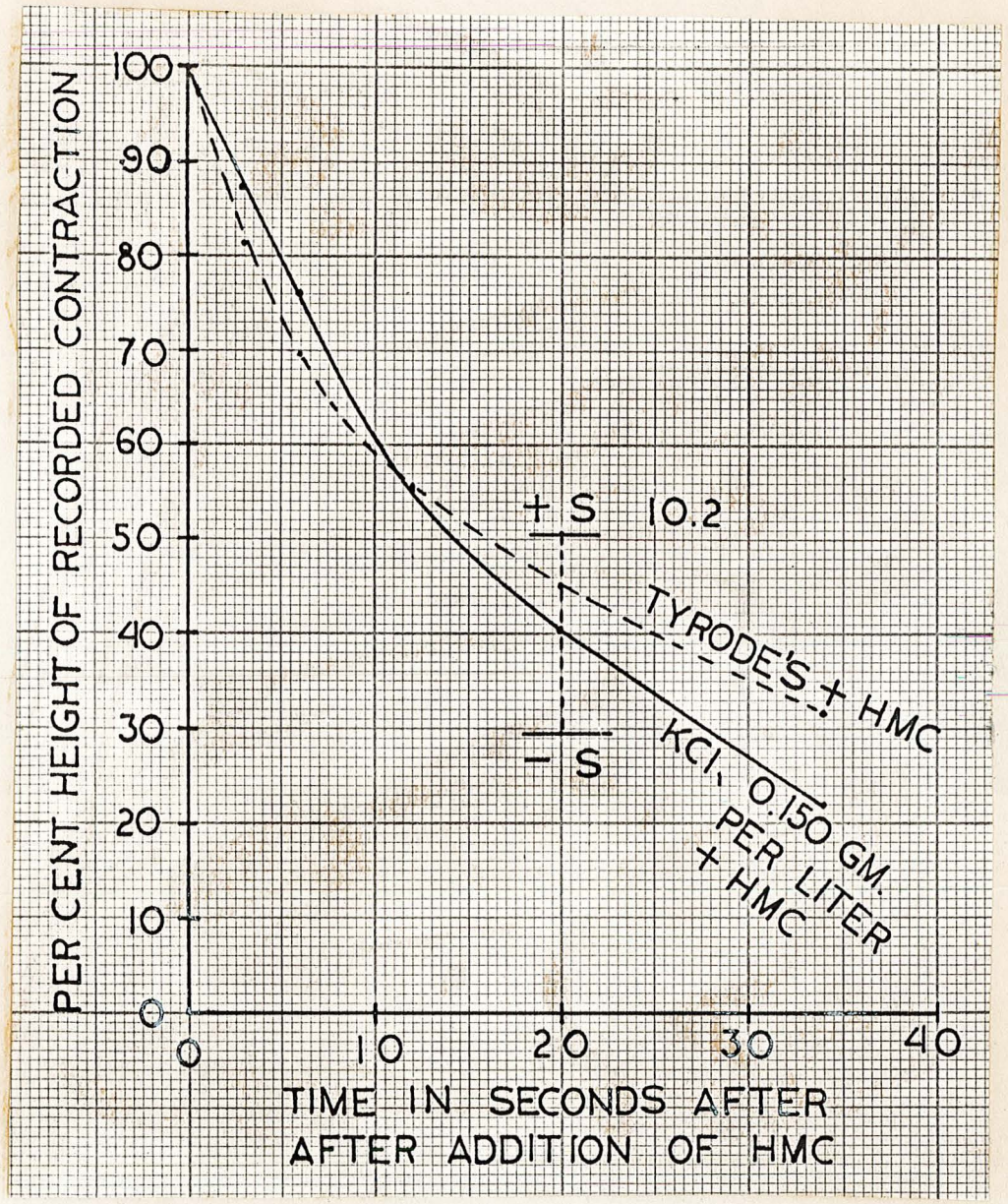



Figure 4. Mean response to HMC of muscles contracting in modified Tyrode's solution containing 0.150 grams of potassium chloride per liter (10 trials). (The response does not differ significantly from that obtained in Tyrode's solution.)

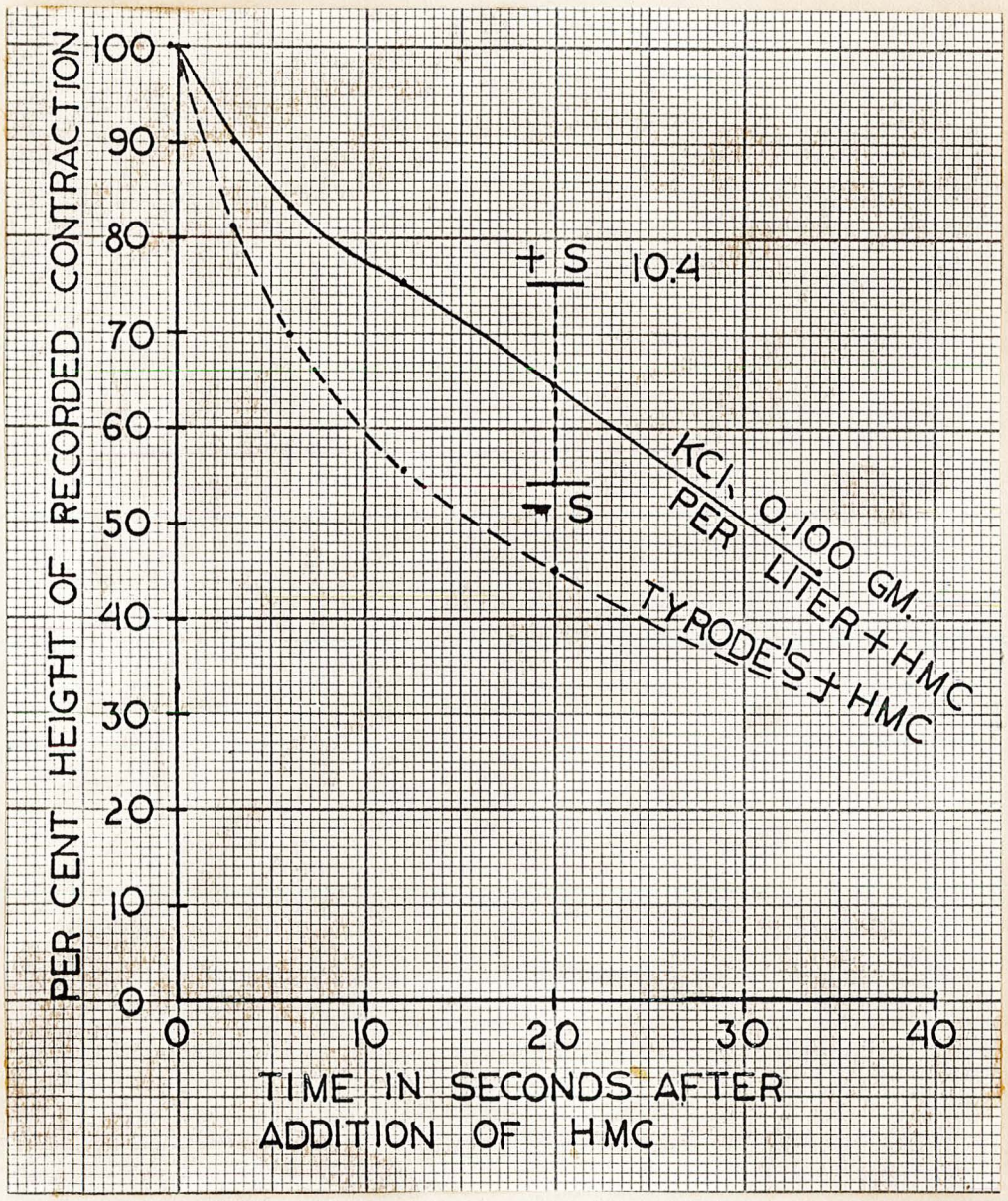



Figure 5. Mean response to HMC of muscles contracting in modified Tyrode's solution containing 0.100 grams of potassium chloride per liter (10 trials). (The difference between the curves is significant at the 0.95 level.)

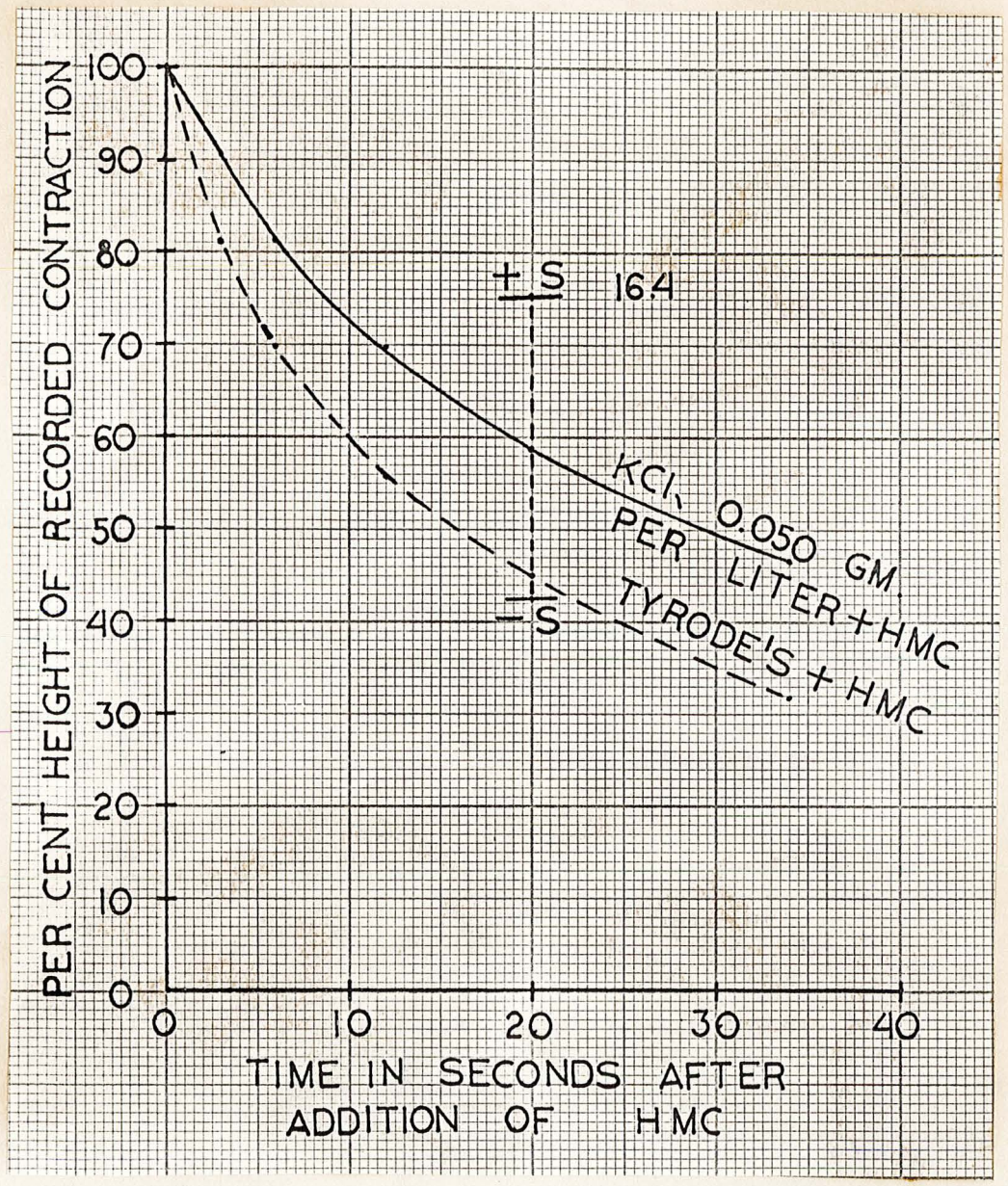



Figure 6. Mean response to HMC of muscles contracting in modified Tyrode's solution containing 0.050 grams of potassium chloride per liter (10 trials). (The difference between the curves is significant at the 0.95 level.)

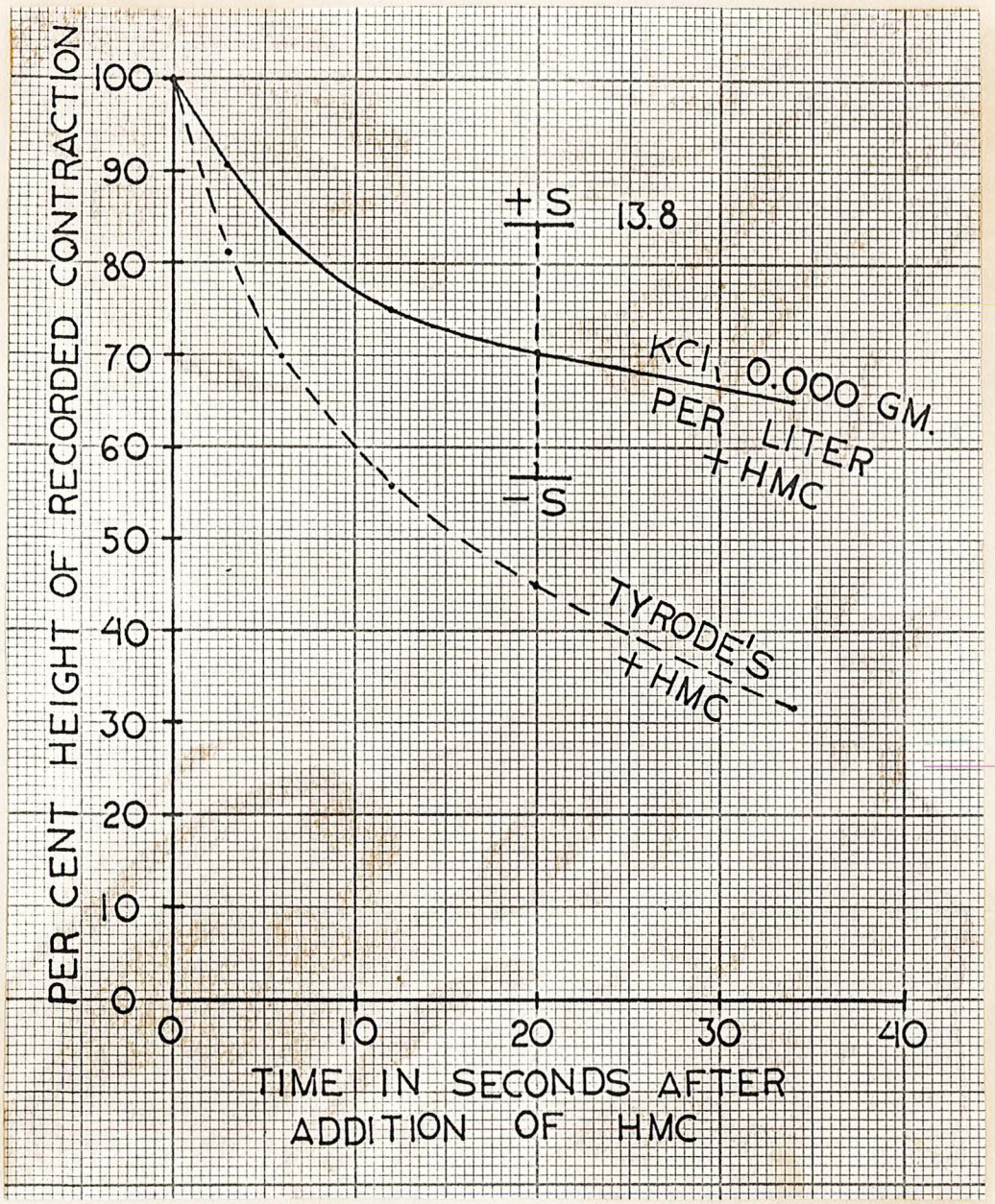



Figure 7. Mean response to HMC of muscles contracting in modified Tyrode's solution containing no potassium (12 trials). (The difference between the curves is significant at the 0.99 level.)

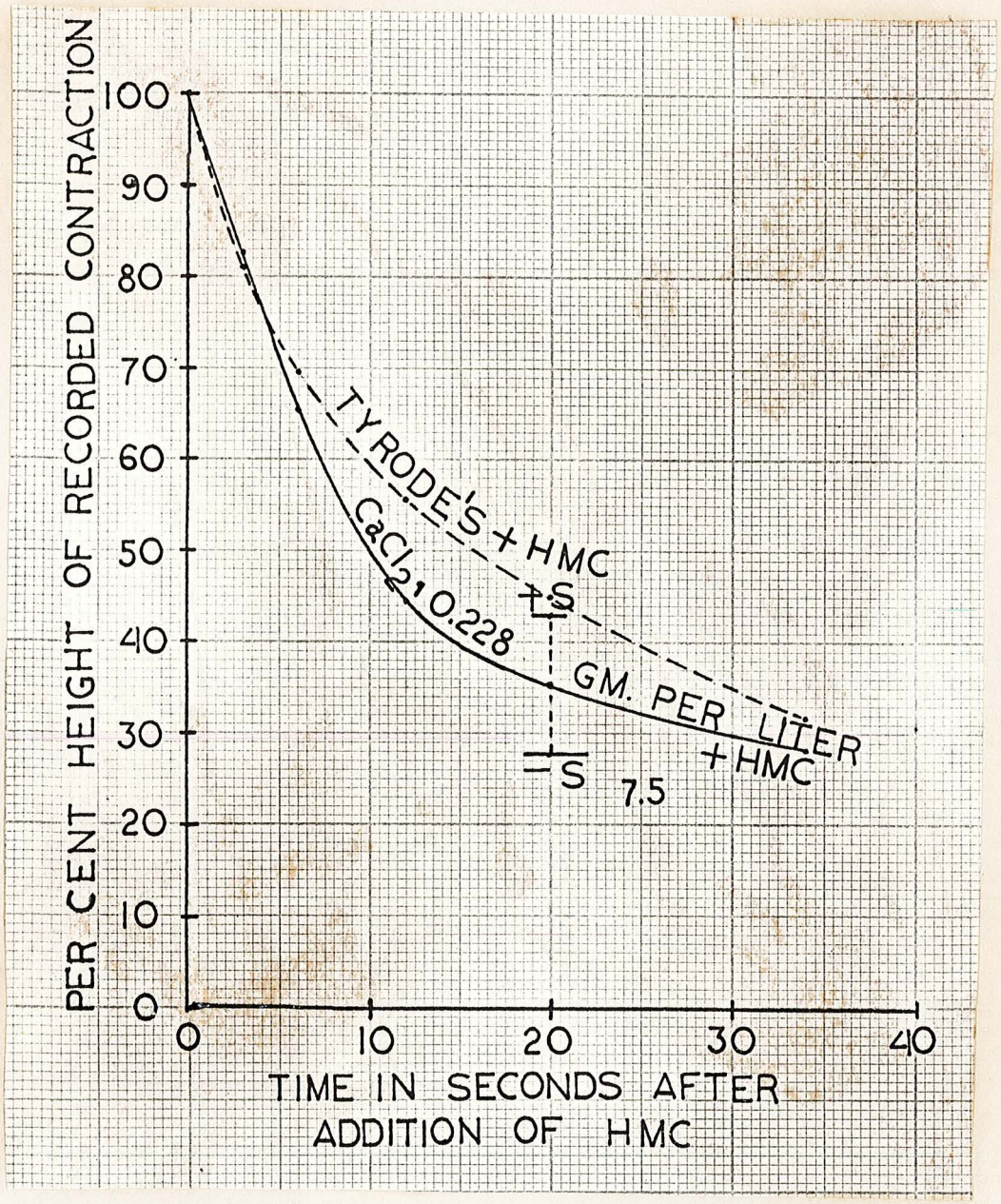



Figure 8. Mean response to HMC of muscles contracting in modified Tyrode's solution containing 0.228 grams of calcium chloride per liter (10 trials). (The difference between the curves is not significant.)

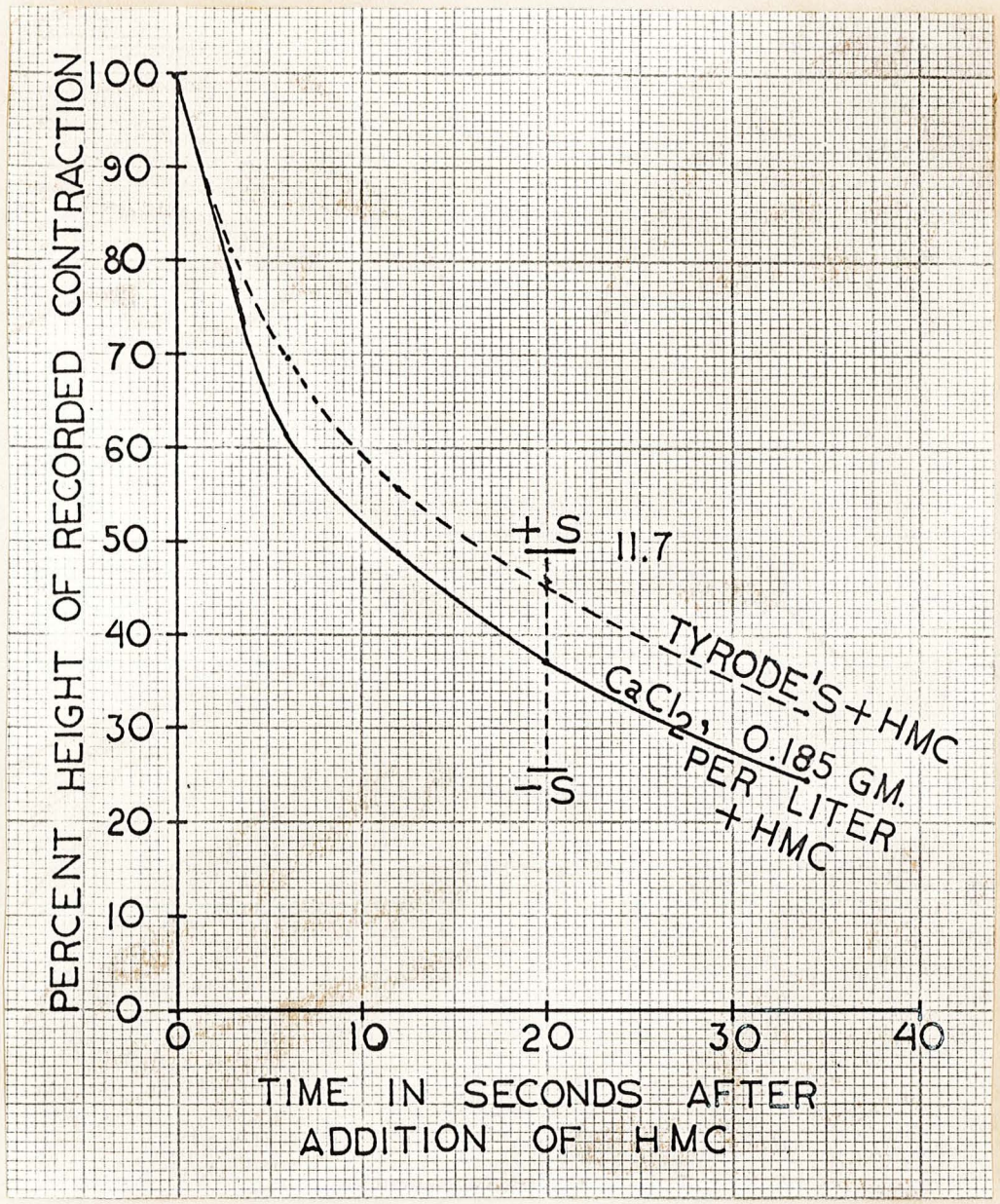



Figure 9. Mean response to HMC of muscles contracting in modified Tyrode's solution containing 0.185 grams of calcium chloride per liter (10 trials). (The difference between the curves is not significant.)

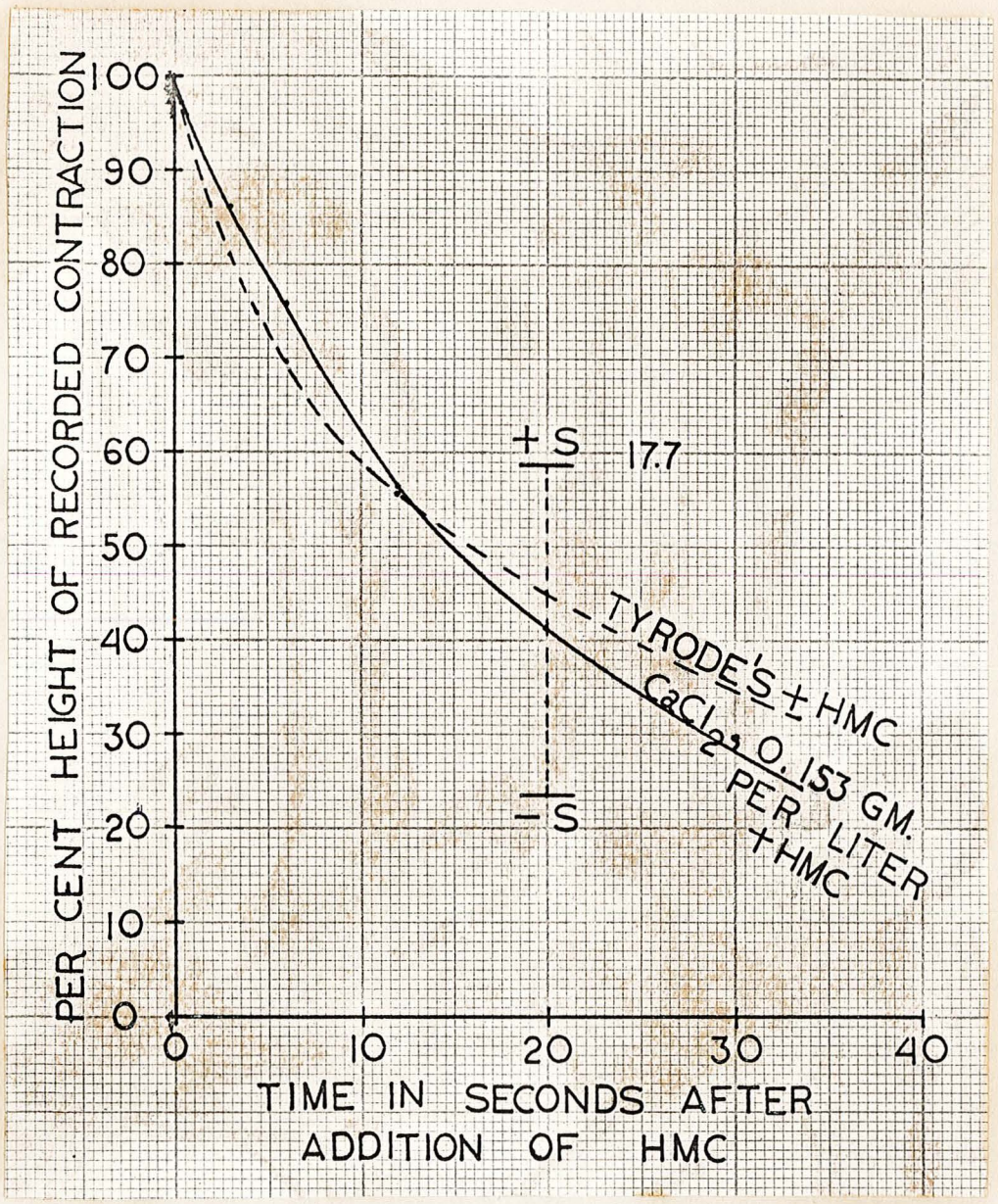



Figure 10. Mean response to HMC of muscles contracting in modified Tyrode's solution containing 0.153 grams of calcium chloride per liter (9 trials). (The response does not differ significantly from that in Tyrode's solution.)

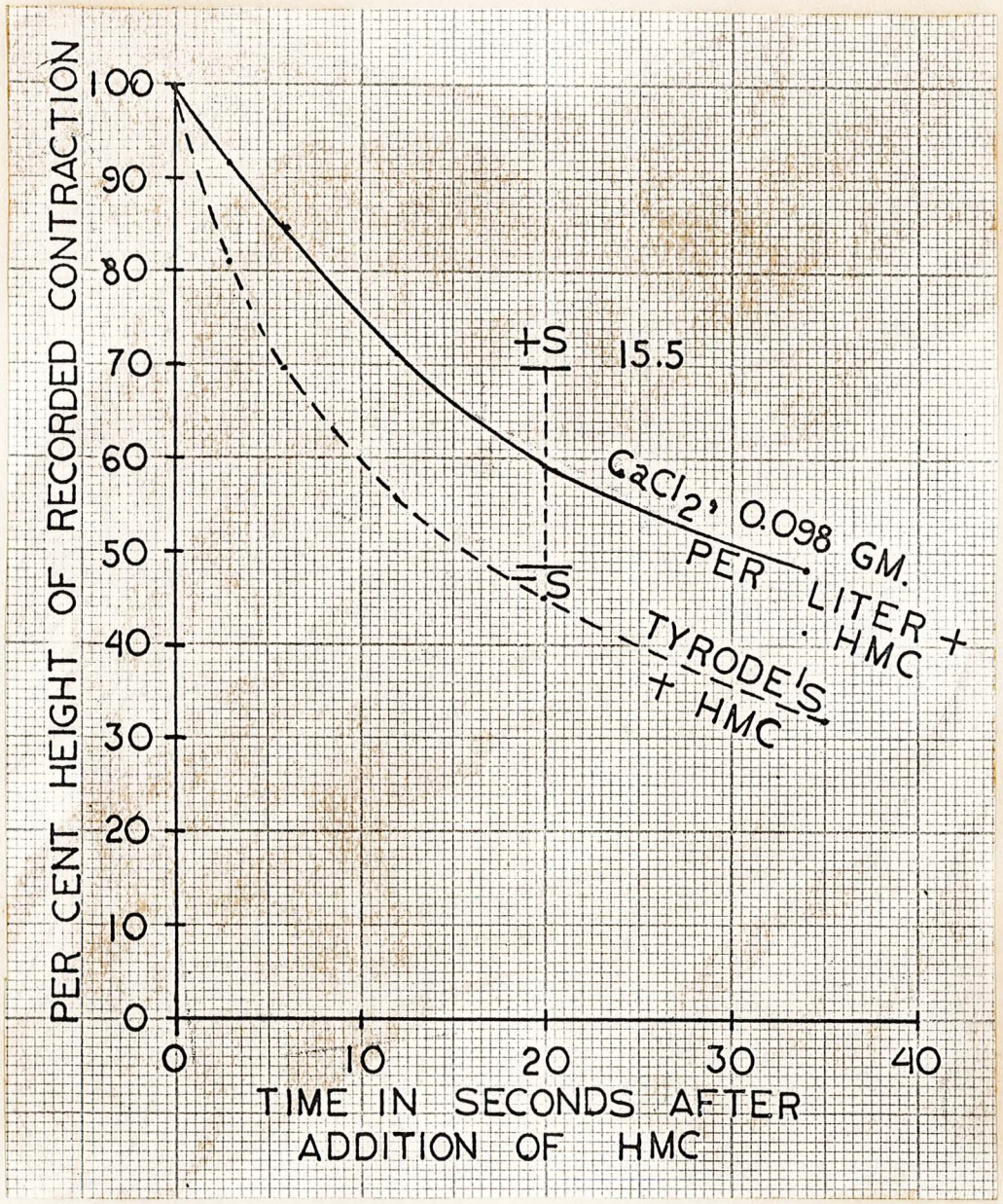



Figure 11. Mean response to HMC of muscles contracting in modified Tyrode's solution containing 0.098 grams of calcium chloride per liter (8 trials). (The difference between the curves is significant at the 0.95 level.)

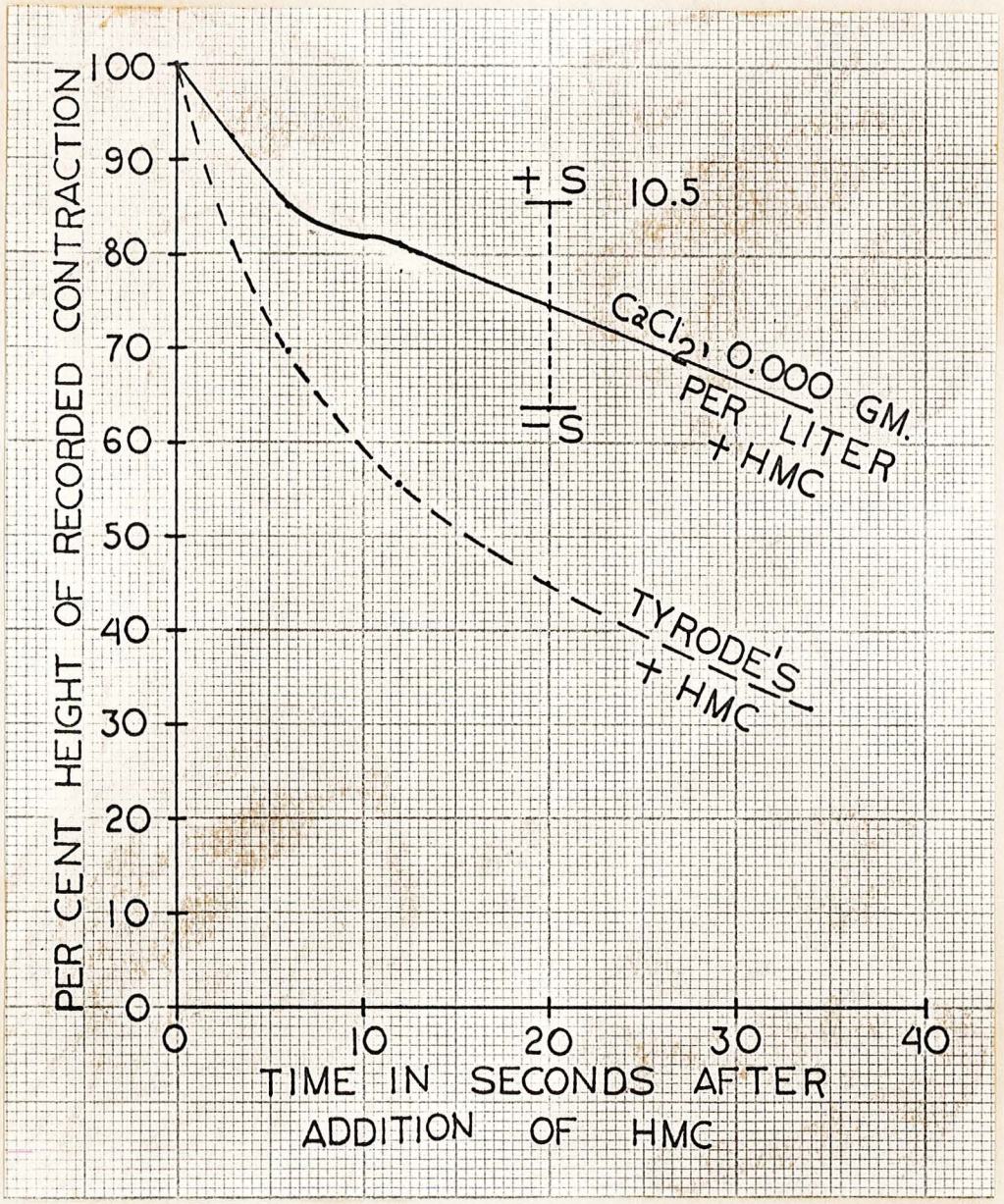



Figure 12. Mean response to HMC of muscles contracting in modified Tyrode's solution containing no calcium (9 trials). (The difference between the curves is significant at the 0.99 level.)

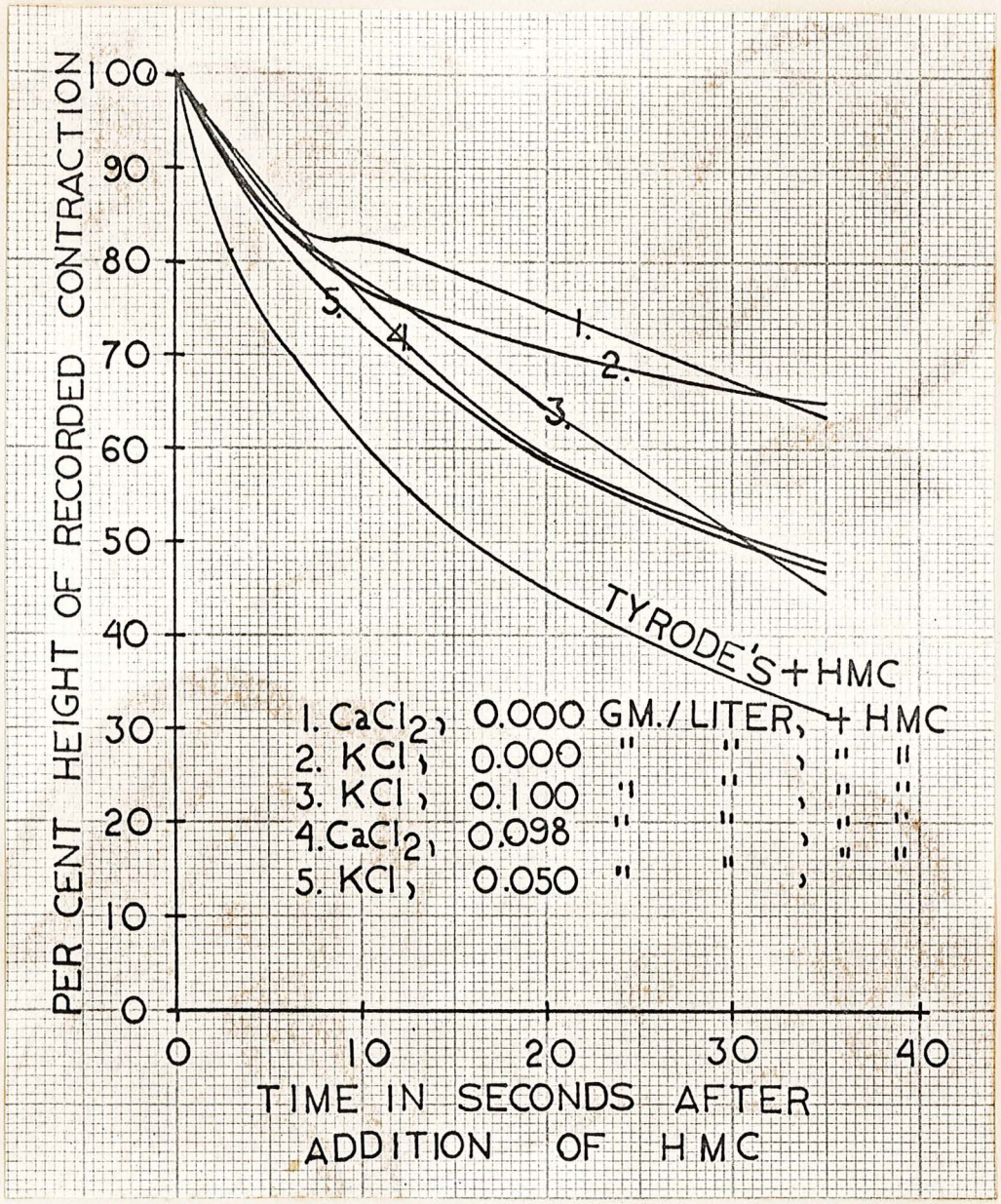



Figure 13. Response curves with significant departures from the response obtained in standard Tyrode's solution.

in potassium, is shown in Figure 14. A state of sustained contraction resulted from the exposure to the high potassium concentration, a state very similar to the contracture caused by excess potassium on the heart (11). This contracture was reversed by addition of HMC after which the muscle returned to previous rhythmic contraction.

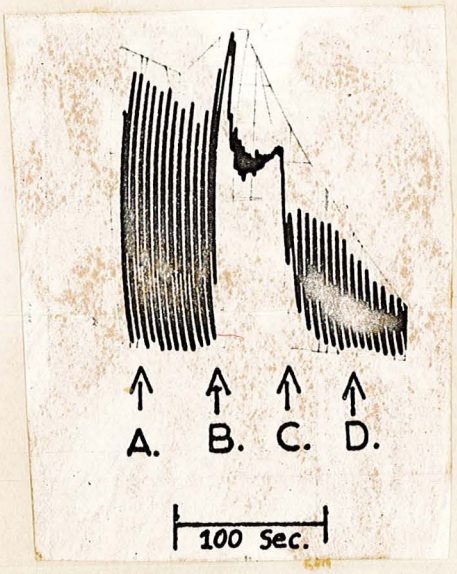



Figure 14. Effect of an eightfold increase in potassium followed by addition of HMC.

A. Normal contractions.
B. Exchange to solution containing eightfold increase in potassium.
C. Addition of 10 milligrams of HMC.
D. Resumption of rhythmic contractions.

Cardiotonic glycosides. As little as 0.025 units of the glycosides of digitalis caused increased contractility of the muscle strips. In a typical experiment this amount was added to the bath. The muscle responded with an increased

height of contraction. Then 5 milligrams of HMC were added and the height decreased to its original value. Another addition of digitalis, 0.05 units, caused another increase in height which was reversed by 10 milligrams of HMC. This and similar experiments were performed which showed digitalis and HMC to be mutually antagonistic in their effects on contraction. In all cases, the glycosides were able to reverse the response to HMC and HMC was able to reverse the response to digitalis. The significance of this antagonism will be discussed in the Discussion part of the thesis.

Comparison with sodium versenate. Less than 10 milligram amounts of sodium versenate lowered the frequency of contraction; otherwise, there appeared to be no response. Amounts greater than 10 milligrams quickly inhibited muscle strips. Muscles contracting in potassium-free Tyrode's solutions were immediately inhibited when 15 milligrams of the chelating agent were added. This inhibition was in direct contrast to the antagonism to HMC provided by potassium-free solutions (Figure 7, page 22).

The attempt to measure the chelating ability of HMC by titration (raw data tabulated in the Appendix) gave the results in Table V. In both cases, the solutions containing HMC needed slightly more titrant than did the water controls. But that difference is insignificant when compared to the

much greater amounts taken by solutions containing sodium versenate.

TABLE V

AVERAGE MILLILITERS OF TITRANT NEEDED TO DEVELOP PERMANENT TURBIDITY

| Experimental solutions | Trial 1, calcium oxalate indicator; sodium oxalate added | Trial 2, calcium tartrate indicator; calcium chloride added |
|---|---|---|
| Water controls | 0.44 | 4.77 |
| Solutions containing HMC | 0.99 | 4.86 |
| Solutions containing sodium versenate | 12.40 | 6.27 |

The differences between the action of HMC and sodium versenate elicited in potassium-free solutions make it improbable that HMC and sodium versenate inhibit smooth muscle by the same mechanism. The paucity of calcium binding shown by HMC in the titration studies adds support to the same conclusion.

# CHAPTER IV

## DISCUSSION

The effects produced by norepinephrine, injected before and after HMC (Figure 2, page 15), were not greatly changed by the bioflavonoid. HMC was unable to diminish the nictitating membrane contractions and pupil dilations resulting from stimulation of cervical sympathetic fibers while phentolamine was able to do so. These results could be taken to indicate that the bioflavonoid does not cause reduced blood pressure by an adrenolytic mechanism. The undiminished nictitating membrane and pupil response also indicate that the bioflavonoid apparently is not a ganglioplegic agent. Hexamethonium, in contrast, completely abolished the effects of preganglionic stimulation.

Since a fall in blood pressure sometimes follows administration of a cholinergic drug, the possibility that HMC might have a similar effect could not be overlooked. However, as shown in the section on results, atropine sulfate, a typical anticholinergic, did not prevent the hypotensive effect of HMC, but did protect against a blood pressure drop when acetylcholine was injected (Figure 2, parts C and D).

The elimination of the mechanisms tested in the <u>in vivo</u> studies suggests that HMC might have a direct action on the muscle cells. The results from the various <u>in vitro</u>

studies support the hypothesis that HMC inhibits contraction by rendering the cell membranes impermeable to potassium efflux or to other ionic shifts occuring with contraction. This conclusion is reached from observing that the solutions or agents which interfere with the effect of HMC are ones which seem to facilitate potassium efflux or produce a decrease in the amount of intracellular potassium.

As commented on by Thomas (12), potassium ion has differing intracellular and extracellular actions on smooth muscle. The presence of intracellular potassium inhibits contraction of cardiac muscle and other types of muscle by reversible competition with calcium for reactive sites on the contractile proteins or on a relaxing factor. Thus, if the concentration of intracellular potassium decreases or calcium increases, contraction will occur. A low concentration of potassium in the external environment stimulates contraction by two possible mechanisms. The first possibility, similar to one proposed by Armitage (13) for cardiac muscle, is one concerned with ionic gradients. The intracellular potassium can migrate outward more easily against the reduced potassium concentration. An alternate possibility is a mechanism similar to that in heart muscle in which decreased extracellular potassium causes increased calcium influx (12). Either mechanism might conceivably reverse the inhibitory effect of HMC.

A reduced concentration of calcium in the external environment decreased the inhibitory effect of HMC. It is believed (12) that calcium plays two different roles in contractile processes. According to this theory, the first role of calcium is to provide a link between cell membrane excitation and contraction. Bound calcium is released from storage sites on the cell membrane by excitation and enters the cell to cause contraction. It does this either by directly activating contractile mechanisms or by inactivating a relaxing system that normally prevents contraction (14). This explains the increased contractility that might result from the augmented calcium influx produced by reduced extracellular potassium. The second role played by calcium is in regulating membrane polarity and permeability. High external calcium concentration reduces spike frequency, potassium efflux, and spontaneous muscle tone by stabilizing the cell membrane (15). Conversely, reduction of the external calcium produces membrane instability resulting in increased spike frequency, increased potassium efflux, a partial depolarization, and increased tonus (16). This stimulating effect, though short in duration, is the probable explanation of low calcium antagonism of the bioflavonoid.

As discussed in the previous paragraphs, both low calcium and low potassium in the external environment cause an increased efflux of potassium and both were shown to

antagonize the effect of HMC (Figure 13, page 28). It is reasonable to speculate that HMC might cause inhibition of muscle by hindering potassium efflux or other ionic shifts, perhaps by decreasing the permeability of the cell membrane.

Cardiotonic glycosides which were found in our work to reverse the effects of HMC are reported by Kahn, et al (8), to cause the amount of intracellular potassium to decrease. This is accomplished by a decrease in the rate of potassium influx following contraction. The glycosides do not appear to change the rate of potassium efflux. This is in agreement with our observations that a change in the handling of potassium by the cell membrane reversed the action of HMC which suggests that the bioflavonoid might be producing a modification of potassium exchange by the cell membrane.

High external potassium concentration causes contracture in many types of muscle including arterial segments (17), intestinal muscle (18), and cardiac muscle (11). A sufficient increase in extracellular potassium depolarizes the muscle membrane. This causes the release of bound calcium which migrates transcellularly resulting in contracture (12). As shown in Figure 14, page 29, HMC reversed the contracture of the eightfold increase of potassium ion, thus allowing the muscle to resume rhythmical contractions, although of a somewhat diminished amplitude. This particular observation is not contradictory to the previous observations

where HMC decreased contraction, but actually substantiates the former conclusion of interference with ionic transport. In the first instance, HMC decreases amplitude of contraction by preventing potassium efflux or calcium shifts, thus interfering with the intracellular contractile mechanisms. In the second instance, which is purely an extreme experimental situation, excess potassium causes apparent depolarization which allows the release of bound calcium resulting in contraction. HMC by the same membrane stabilization which prevents efflux of potassium in Tyrode's solution allows relaxation of the muscle by preventing the excess potassium from depolarizing.

That these conclusions are possible is supported by Steinberger, et al (19), who provide evidence that bioflavonoids can alter ionic transport. They found that peritoneal absorption of isotonic sodium chloride was substantially decreased in rats treated with phosphorylated hesperidin. They also reported that the bioflavonoid caused decreased permeability of mouse connective tissue membranes in vitro. Additional evidence is provided by Call and Patterson (20) who reported that a lemon bioflavonoid complex protected rats against normally lethal doses of ephedrine and epinephrine. They concluded that the protection was probably caused by decreased absorption of the toxic agents. It is

very tempting to assume a relationship between the decreased transport of ions and drugs and the decreased permeability to potassium hypothesized previously.

# CHAPTER V

## CONCLUSION

This thesis reports studies on the mechanisms underlying the blood pressure reduction and smooth muscle inhibition caused by the bioflavonoid hesperidin methyl chalcone. Experiments on intact circulatory systems and nictitating membranes of cats ruled out adrenolytic, ganglioplegic, and cholinergic mechanisms for the lowering of blood pressure.

Studies on smooth muscle *in vitro* (rabbit ileum) showed that the inhibition caused by the bioflavonoid was reversible by low potassium or low calcium concentration in the surrounding fluid. The bioflavonoid reversed the contracture caused by an eightfold increase of potassium in the external environment and allowed resumption of rhythmic contraction. Cardiotonic glycosides and HMC were found to be mutually antagonistic in their effects on contraction.

It is concluded that HMC may have its smooth muscle depressant effect by virtue of causing decreased membrane permeability to ions or by interfering with membrane transport mechanisms.

# BIBLIOGRAPHY

# BIBLIOGRAPHY


1. Vogin, E. E. A review of bioflavonoids, Amer. J. Pharm. 132: 363-82, October 1960.

2. Kikuchi, K., H. Hirata, M. Komatsu, H. Yokotani, H. Nishida, and Y. Aramaki. Pharmacological studies on methyl hesperidin. IV. Effects on blood pressure, isolated perfused heart, and other smooth muscles, Nippon Yakurigaku Zasshi 56:1387-402, 1960: Chem. Abstr. 56:6621b., 1962.

3. Aramaki, Y., H. Yokotani, I. Ishikawa, M. Baba, and M. Komatsu. Biological activity of methylhesperidin, Shinryo 10:727-35, 1957: Chem. Abstr. 53: 8435b., 1959.

4. Riedesel, C. C. Unpublished screening studies, University of the Pacific, Department of Physiology-Pharmacology.

5. Joseph, G. Some chemical aspects of the flavonoids, Sunkist Growers Research Bulletin D-2762.

6. Hoff, H. E., and L. A. Geddes. Experimental Physiology. Texas Medical Center, Baylor University, page 18a, 1962.

7. Martin, E. E., and E. F. Cook (eds.) Remington's Practice of Pharmacy, 12th edition. Mack Publishing Co., Easton, Pa., 364 pp., 1961.

8. Kahn, J. B., E. Eakin, and D. E. Levi. Effects of ouabain and calcium on potassium balance in isolated guinea pig ventricle, Amer. J. Physiol. 203: 1130-34, December 1962.

9. Ebashi, S. Calcium binding and relaxation in the actomyosin system, J. Biochem. (Tokyo) 48:150-51; 1960: Chem. Abstr. 55:2747g, 1961.

10. Waugh, W. H. The role of calcium in contractile excitation of vascular smooth muscle by epinephrine and potassium, Circulat. Res. 11:927-94, December 1962.

11. Niedergerke, R. The potassium chloride contracture of the heart and its modification by calcium, J. Physiol. (Lond.) 134:584-99, December 1956.

12. Thomas, L. J. Increase of labeled calcium uptake in heart muscle during potassium lack contracture, J. Gen. Physiol. 43:1193-1206, 1960.

13. Armitage, A. The influence of potassium concentration on the action of quinidine and of some anti-malerial substances on cardiac muscle, Brit. J. Pharmacol. 12:74-78, 1957.

14. Winegrad, S. The possible role of calcium in excitation-contraction coupling of heart muscle, Circulation 24:523-29, 1961.

15. Hurwitz, L., F. Battle, and G. B. Weiss. Action of the calcium antagonists cocaine and ethanol on contraction and potassium efflux of smooth muscle, J. Gen. Physiol. 46:315-32, November 1962.

16. Hurwitz, L., B. Tinsley, and F. Battle. Dissociation of contraction and potassium efflux in smooth muscle, Amer. J. Physiol. 199:107-111, July 1960.

17. Hinke, J. A. M., and M. L. Wilson. Effects of electrolytes on contractility of artery segments in vitro, Amer. J. Physiol. 203:1161-66, December 1962.

18. Bulbring, E. Electrical activity in intestinal smooth muscle, Physiol. Rev. 42 Suppl. 5:160-72, 1962.

19. Steinberger, E., W. J. Dixon, and R. A. Jones. Effect of phosphorylated hesperidin and hyaluronidase on absorption from peritoneal cavity, Proc. Soc. Exp. Biol. Med. 97:598, 1958.

20. Call, T. G., and J. A. Patterson. Protective activity of some flavonoid materials against the acute toxicity of adrenergic compounds to rats, J. Amer. Pharm. Ass. (Sci.) 47:847-48, December 1958.

# APPENDIX

# APPENDIX

## I. DRUGS AND REAGENTS USED

Bioflavonoid.

Hesperidin Methyl Chalcone D-3442. Commercial product.

Reagent Grade Materials, used in physiological solutions.

Sodium Chloride.

Potassium Chloride.

Calcium Chloride, Dihydrate.

Magnesium Chloride, Hexahydrate.

Monobasic Sodium Phosphate, Monohydrate.

Sodium Bicarbonate.

Dextrose.

Reagent Grade Materials, used in titration studies.

Disodium Ethylenediaminetetraacetate. (Sodium Versenate).

Sodium Oxalate.

Sodium Potassium Tartrate, Tetrahydrate. (Rochelle Salt--N.F. Grade).

U. S. P. Grade Drugs.

Acetylcholine Chloride.

Norepinephrine Hydrochloride.

Posterior Pituitary Extract.

Phentolamine Hydrochloride.

Hexamethonium Chloride.

Epinephrine Hydrochloride.

Pentobarbital Sodium.

Digitalis Glycosides. (Digiglusin--Lilly).

Atropine Sulfate.

## II. IN VIVO EXPERIMENTS

### Blood Pressure Studies

The data for these experiments are given in outline form. The results of injection of various compounds are presented in the order injected. Blood pressure changes are reported as percentages with the pressure at the time of injection taken to be 100 per cent. All of the original records are in the files of the Department of Physiology-Pharmacology.

I. 9/24/63. The subject of this experiment was a 4 kilogram male cat. Blood pressure was recorded on an ink writing kymograph by a "U" tube mercury manometer.

a. Challenge Doses.

1. Norepinephrine, 0.1 microgram (mcg.) per kilogram (Kg.), caused a 50 per cent rise.

2. Norepinephrine, 0.2 mcg./Kg., caused a 44 per cent rise.

3. Acetylcholine (ACh), 1.0 mcg./Kg., caused a 68 per cent fall.

4. ACh, 2.0 mcg./Kg., caused a 94 per cent fall.

b. Experimental Doses.

1. HMC, 25 milligrams (mg.)/Kg., caused a 58 per cent fall.

2. HMC, 25 mg./Kg., caused a 57 per cent fall.

3. HMC, 50 mg./Kg., caused an 81 per cent fall.

4. Atropine sulfate, 0.5 mg./Kg., caused a very transient fall in pressure, 27 per cent.

5. HMC, 50 mg./Kg. The pressure fell from 93 to 40 millimeters (mm.) of mercury (Hg) at ninety seconds after injection. While it was still falling, the following dose of atropine was given.

6. Atropine sulfate, 0.5 mg./Kg. The pressure continued to fall and fell to 28 mm. Hg within ninety seconds after this injection and then held steady, a 70 per cent total fall after HMC.

7. Atropine sulfate, 1.0 mg./Kg., given nine minutes from the previous dose; the pressure fell from 20 to 8 mm. Hg.

8. Norepinephrine, 0.2 mcg./Kg., caused a 46 per cent rise.

9. A series of massive doses of norepinephrine was given (85 mcg. total dose).

10. ACh, 2.0 mcg./Kg., caused no change in pressure, showing that the atropine was still effective.

11. HMC, 100 mg./Kg. The pressure fell from 35 to 4 mm. Hg. Although the atropine was still effective, it did not reverse the hypotensive action of the bioflavonoid.

II. 10/31/63. The subject of this experiment was a 3.5 kilogram male cat. Arterial pressure was recorded by transducer from the left femoral artery, and an electrocardiogram was recorded simultaneously to measure heart rate.

a. Challenge Doses.

1. ACh, 1.0 mcg./Kg., causes a 66 per cent fall and the heart rate decreased.

2. Norepinephrine, 0.1 mcg./Kg., caused a 22 per cent fall in pressure. This unexpected result might be caused by ACh left in the vein from the previous injection.

3. Norepinephrine, 0.1 mcg./Kg., caused a 5 per cent rise.

4. Norepinephrine, 0.2 mcg./Kg., caused a 16 per cent rise and the heart rate was unchanged.

5. Norepinephrine, 1.0 mcg./Kg., caused a 37.5 per cent rise.

6. Epinephrine, 0.1 mcg./Kg., caused an 11 per cent rise in pressure and an increased heart rate.

7. Epinephrine, 0.2 mcg./Kg., caused a 26 per cent rise.

8. Pentobarbital sodium, 12.5 mg., caused a 30 per cent fall in pressure. This is the expected vascular response to barbiturate injection.

b. Experimental Doses.

1. HMC, 25 mg./Kg., caused a 19 per cent fall in pressure with an unchanged heart rate.

2. HMC, 50 mg./Kg., caused a 43 per cent fall with no change in heart rate.

3. Norepinephrine, 0.2 mcg./Kg., caused a 19 per cent rise.

4. Norepinephrine, 1.0 mcg./Kg., caused a 38 per cent rise. The heart rate was unchanged.

5. Epinephrine, 0.2 mcg./Kg., caused a 58 per cent rise.

6. ACh, 1.0 mcg./Kg., caused a 63 per cent fall.

7. Pituitary extract, 10 units, caused a 78 per cent rise with no change in heart rate.

8. HMC, 50 mg./Kg., caused a 62 per cent fall.

9. Hexamethonium chloride, 12 mg., caused a 32 per cent fall. There was a small decrease in heart rate.

III. 11/19/63. A 2.7 Kg. female cat was the subject of this experiment. Nictitating membrane studies were performed simultaneously with the blood pressure work.

a. Challenge Doses.

1. ACh, 1.0 mcg./Kg., caused a 71 per cent fall.

2. Norepinephrine, 0.2 mcg./Kg., caused a 14 per cent rise.

3. Pentobarbital sodium, 10 mg., caused a transient fall in pressure.

4. Pituitary extract, 2.5 units, caused a 41 per cent rise.

b. Experimental Doses.

1. Atropine sulfate, 0.5 mg./Kg., caused a very slight and transient rise in pressure.

2. ACh, 1.0 mcg./Kg., caused an 11 per cent fall in comparison with a 71 per cent fall before atropine.

3. HMC, 50 mg./Kg., caused a 51 per cent fall.

4. (Approximately 3-1/2 minutes after 3.) HMC, 50 mg./Kg., caused a 30 per cent fall.

5. (Approximately 5 minutes after 4.) HMC, 50 mg./Kg., caused an 18 per cent fall.

6. Norepinephrine, 0.2 mcg./Kg., caused a 25 per cent rise.

7. Pituitary extract, 2.5 units, caused a 27 per cent rise.

8. Hexamethonium chloride, 12 mg. total dose, caused a gradual decline in blood pressure.

Nictitating Membrane Studies

The significant findings from this work are presented in the chapter on Results. All of the Physiograph records are on file in the Department of Physiology-Pharmacology.

## III. IN VITRO STUDIES

The Physiograph records (on file in the Department of Physiology-Pharmacology) were handled in the following manner. The heights of recorded contraction were measured and converted into percentages with the height at the time of addition of HMC taken to be 100 per cent. The values for each member of a series were plotted as line graphs versus time in seconds after addition of HMC. The mean per cent height of recorded contractions were calculated at 3, 6, 12, 20, and 34 seconds after addition of the bioflavonoid. The significance of the deviation of each response from that in Tyrode's solution was calculated at the 20 second time value. The Student-t test was used. These curves and comparisons are presented in the chapter on Results. The raw data from which the line graphs were drawn are compiled in this section.

Table 1

Data from 12 forty-second time periods of muscle contracting in Tyrode's solution. The origin of each run was chosen arbitrarily.

| D* | H | % | S | D | H | % | S | D | H | % | S |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 12/15/63 | 4.03 | 100 | 0 | 2/4/64 | 3.06 | 100 | 0 | 2/20/64 | 2.20 | 100 | 0 |
| A. | 3.88 | 96 | 10 | A. | 3.20 | 105 | 10 | A. | 2.40 | 109 | 10 |
| | 4.10 | 102 | 20 | | 3.42 | 112 | 20 | | 2.08 | 95 | 20 |
| | 4.10 | 102 | 30 | | 2.96 | 97 | 30 | | 2.38 | 108 | 30 |
| | 4.20 | 104 | 40 | | 3.13 | 102 | 40 | | 1.90 | 87 | 40 |
| B. | 3.78 | 100 | 0 | B. | 2.20 | 100 | 0 | B. | 3.82 | 100 | 0 |
| | 4.10 | 109 | 10 | | 1.95 | 89 | 10 | | 3.65 | 96 | 10 |
| | 3.85 | 102 | 20 | | 1.89 | 86 | 20 | | 3.32 | 87 | 20 |
| | 3.92 | 104 | 30 | | 1.99 | 91 | 30 | | 3.20 | 84 | 30 |
| | 3.69 | 98 | 40 | | 1.65 | 75 | 40 | | 3.50 | 92 | 40 |
| C. | 3.50 | 100 | 0 | 2/13/64 | 2.25 | 100 | 0 | 3/16/64 | 2.50 | 100 | 0 |
| | 3.33 | 95 | 10 | A. | 2.05 | 91 | 10 | A. | 2.56 | 102 | 10 |
| | 3.20 | 91 | 20 | | 2.60 | 116 | 20 | | 2.60 | 104 | 20 |
| | 3.18 | 91 | 30 | | 2.50 | 111 | 30 | | 2.60 | 104 | 30 |
| | 3.38 | 96 | 40 | | 2.18 | 97 | 40 | | 2.70 | 108 | 40 |
| D. | 3.30 | 100 | 0 | B. | 3.88 | 100 | 0 | B. | 2.30 | 100 | 0 |
| | 2.98 | 90 | 10 | | 4.00 | 103 | 10 | | 2.30 | 100 | 10 |
| | 3.35 | 102 | 20 | | 3.12 | 81 | 20 | | 2.30 | 100 | 20 |
| | 3.25 | 99 | 30 | | 3.55 | 92 | 30 | | 2.32 | 101 | 30 |
| | 3.25 | 99 | 40 | | 4.22 | 109 | 40 | | 2.20 | 96 | 40 |

The mean per cent heights of contraction were 99.8, 99.3, 98.6, 98.2, and 98.0 at 3, 6, 12, 20, and 34 seconds, respectively.

*D = date and experiment letter on the original Physiograph record; H = measured height of recorded contraction in centimeters; % = calculated per cent height of recorded contraction; S = time in seconds after the arbitrary starting point.

Table 2

Response to HMC of muscles contracting in Tyrode's solution

| D* | H | % | S | D | H | % | S |
|---|---|---|---|---|---|---|---|
| 2/4/64 | 2.05 | 100 | 0 | 2/4/64 | 3.65 | 100 | 0 |
| 1. | 1.30 | 64 | 5 | 17. | 2.25 | 62 | 6 |
| | 0.92 | 45 | 11 | | 1.94 | 53 | 17 |
| | 0.68 | 33 | 32 | | 1.75 | 48 | 30 |
| | 0.58 | 39 | 39 | | 1.55 | 42 | 37 |
| 5. | 2.83 | 100 | 0 | 18. | 3.30 | 100 | 0 |
| | 2.25 | 80 | 6 | | 2.00 | 61 | 3 |
| | 1.56 | 55 | 14 | | 1.62 | 49 | 12 |
| | 1.22 | 43 | 22 | | 1.38 | 42 | 20 |
| | 0.72 | 25 | 27 | | 1.32 | 40 | 27 |
| | 0.52 | 18 | 31 | | 1.18 | 36 | 35 |
| | 0.36 | 13 | 41 | | | | |
| | | | | 19. | 2.68 | 100 | 0 |
| 15. | 2.50 | 100 | 0 | | 1.90 | 71 | 4 |
| | 2.35 | 94 | 9 | | 1.38 | 52 | 11 |
| | 2.20 | 88 | 12 | | 1.30 | 49 | 26 |
| | 1.78 | 71 | 21 | | 1.15 | 43 | 39 |
| | 1.55 | 62 | 30 | | | | |
| | 1.35 | 54 | 33 | 20. | 2.20 | 100 | 0 |
| | 1.28 | 51 | 39 | | 1.04 | 47 | 9 |
| | | | | | 0.78 | 35 | 14 |
| 16. | 3.30 | 100 | 0 | | 0.60 | 27 | 24 |
| | 2.05 | 62 | 4 | | 0.56 | 25 | 29 |
| | 1.88 | 57 | 17 | | 0.40 | 18 | 35 |
| | 1.50 | 46 | 24 | | | | |
| | 1.20 | 36 | 28 | | | | |
| | 1.04 | 32 | 34 | | | | |

Table 2 (continued)

| D | H | % | S | D | H | % | S |
|---|---|---|---|---|---|---|---|
| 2/4/64 | 3.60 | 100 | 0 | 2/4/64 | 2.70 | 100 | 0 |
| 21. | 2.40 | 67 | 7 | 23. | 1.75 | 65 | 3 |
| | 1.60 | 45 | 10 | | 1.22 | 45 | 12 |
| | 1.46 | 41 | 20 | | 0.88 | 33 | 20 |
| | 0.88 | 24 | 33 | | 0.70 | 26 | 25 |
| | | | | | 0.60 | 22 | 35 |
| 22. | 2.25 | 100 | 0 | | | | |
| | 1.85 | 82 | 10 | 24. | 2.24 | 100 | 0 |
| | 1.10 | 49 | 18 | | 1.89 | 84 | 4 |
| | 0.72 | 32 | 22 | | 1.30 | 58 | 9 |
| | 0.69 | 31 | 34 | | 1.07 | 48 | 18 |
| | | | | | 0.92 | 41 | 23 |
| | | | | | 0.78 | 35 | 24 |
| | | | | | 0.60 | 27 | 33 |
| | | | | | 0.50 | 22 | 42 |

The mean per cent heights of contraction were 81.0, 69.7, 45.0, and 31.7 at 3, 6, 12, 20, and 34 seconds, respectively.

*D = date and experiment number on the original Physiograph record; H = measured height of recorded contraction in centimeters; % = per cent height of recorded contraction; S = time in seconds after addition of HMC.

Table 3

Response to HMC of muscles contracting in modified Tyrode's solution (0.150 grams potassium chloride per liter)

| D* | H | % | S | D | H | % | S |
|---|---|---|---|---|---|---|---|
| 2/13/64 I | 2.43 | 100 | 0 | 2/13/64 II | 3.10 | 100 | 0 |
| 6. | 1.90 | 78 | 4 | 2. | 2.02 | 65 | 9 |
| | 1.23 | 51 | 10 | | 1.18 | 39 | 16 |
| | 0.97 | 40 | 15 | | 1.08 | 35 | 20 |
| | 0.77 | 32 | 20 | | 0.64 | 21 | 24 |
| | 0.48 | 20 | 26 | | 0.52 | 17 | 28 |
| | 0.42 | 17 | 31 | | 0.40 | 13 | 35 |
| | 0.37 | 15 | 39 | | | | |
| | | | | 3. | 3.50 | 100 | 0 |
| 7. | 2.26 | 100 | 0 | | 2.72 | 78 | 9 |
| | 1.85 | 82 | 8 | | 2.25 | 64 | 18 |
| | 1.19 | 53 | 10 | | 1.83 | 52 | 23 |
| | 0.81 | 36 | 19 | | 1.52 | 43 | 29 |
| | 0.63 | 28 | 21 | | 1.20 | 34 | 33 |
| | 0.38 | 17 | 37 | | 0.92 | 24 | 40 |
| 8. | 2.72 | 100 | 0 | 4. | 3.16 | 100 | 0 |
| | 2.52 | 93 | 6 | | 1.75 | 55 | 9 |
| | 1.00 | 37 | 16 | | 1.20 | 38 | 21 |
| | 0.75 | 28 | 21 | | 1.00 | 32 | 30 |
| | 0.58 | 21 | 31 | | 0.72 | 23 | 39 |
| | 0.50 | 18 | 41 | | | | |
| | | | | 5. | 2.85 | 100 | 0 |
| 9. | 2.58 | 100 | 0 | | 2.58 | 90 | 3 |
| | 1.87 | 73 | 4 | | 1.56 | 55 | 10 |
| | 1.35 | 52 | 10 | | 0.90 | 32 | 22 |
| | 1.20 | 47 | 15 | | 0.70 | 25 | 29 |
| | 0.99 | 38 | 21 | | 0.58 | 20 | 44 |
| | 0.73 | 28 | 27 | | | | |
| | 0.60 | 23 | 34 | 6. | 2.55 | 100 | 0 |
| | | | | | 1.60 | 63 | 4 |
| 2/13/64 II | 3.67 | 100 | 0 | | 1.20 | 47 | 14 |
| 1. | 2.80 | 76 | 7 | | 0.99 | 31 | 25 |
| | 2.10 | 57 | 21 | | 0.61 | 24 | 33 |
| | 1.50 | 41 | 24 | | 0.50 | 20 | 40 |
| | 1.02 | 28 | 28 | | | | |
| | 0.70 | 19 | 33 | | | | |
| | 0.49 | 13 | 37 | | | | |

The mean per cent heights of contraction were 87.3, 76.2, 55.3, 40.1, and 21.8 at 3, 6, 12, 20, and 34 seconds, respectively.

*For key, see Table 2.

Table 4

Response to HMC of muscles contracting in modified Tyrode's solution (0.100 grams potassium chloride per liter)

| D* | H | % | S | D. | H | % | S |
|---|---|---|---|---|---|---|---|
| 2/13/64$_{II}$ | 2.32 | 100 | 0 | 4. | 4.18 | 100 | 0 |
| 7. | 1.83 | 79 | 16 | | 3.05 | 73 | 3 |
| | 1.70 | 74 | 33 | | 2.50 | 60 | 13 |
| | 1.50 | 65 | 39 | | 2.00 | 48 | 23 |
| | | | | | 1.40 | 33 | 28 |
| 8. | 2.50 | 100 | 0 | | 1.06 | 25 | 38 |
| | 1.90 | 76 | 4 | | | | |
| | 1.60 | 64 | 17 | 5. | 3.20 | 100 | 0 |
| | 1.43 | 57 | 22 | | 2.62 | 82 | 4 |
| | 1.10 | 44 | 27 | | 2.30 | 72 | 16 |
| | 0.97 | 39 | 31 | | 2.00 | 63 | 29 |
| | 0.83 | 33 | 35 | | 1.70 | 53 | 39 |
| 2/13/64$_{III}$ | 3.43 | 100 | 0 | 6. | 3.30 | 100 | 0 |
| 1. | 3.20 | 93 | 4 | | 2.90 | 88 | 5 |
| | 2.62 | 76 | 6 | | 2.42 | 73 | 14 |
| | 2.50 | 73 | 10 | | 2.18 | 66 | 24 |
| | 1.50 | 44 | 21 | | 1.88 | 57 | 30 |
| | 0.80 | 23 | 24 | | 1.60 | 49 | 35 |
| | 0.72 | 21 | 29 | | | | |
| | 0.47 | 14 | 43 | 7. | 2.80 | 100 | 0 |
| | | | | | 2.00 | 71 | 13 |
| 2. | 4.05 | 100 | 0 | | 1.74 | 62 | 21 |
| | 3.88 | 96 | 9 | | 1.38 | 49 | 29 |
| | 3.31 | 82 | 17 | | 1.08 | 38 | 35 |
| | 2.35 | 58 | 26 | | | | |
| | 1.80 | 45 | 34 | 8. | 2.58 | 100 | 0 |
| | | | | | 2.10 | 81 | 10 |
| 3. | 4.42 | 100 | 0 | | 1.82 | 70 | 25 |
| | 3.38 | 77 | 7 | | 1.62 | 64 | 35 |
| | 2.95 | 67 | 17 | | | | |
| | 2.70 | 61 | 22 | | | | |
| | 2.03 | 46 | 28 | | | | |
| | 1.40 | 32 | 36 | | | | |

The mean per cent heights of contraction were 90.0, 83.2, 75.2, 64.7, and 45.0 at 3, 6, 12, 20, and 34 seconds, respectively.

*For key, see Table 2.

Table 5

Response to HMC of muscles contracting in modified Tyrode's solution (0.050 grams potassium chloride per liter)

| D* | H | % | S | D | H | % | S |
|---|---|---|---|---|---|---|---|
| 2/13/64$_I$ | 3.22 | 100 | 0 | 2/20/64$_I$ | 2.50 | 100 | 0 |
| 1. | 2.60 | 81 | 11 | 3. | 1.56 | 62 | 5 |
| | 2.45 | 76 | 24 | | 1.35 | 54 | 11 |
| | 2.00 | 62 | 34 | | 1.10 | 44 | 17 |
| | | | | | 0.97 | 39 | 24 |
| 2. | 3.37 | 100 | 0 | | 0.88 | 35 | 30 |
| | 2.80 | 83 | 12 | | 0.75 | 30 | 43 |
| | 2.53 | 75 | 23 | | | | |
| | 1.90 | 56 | 28 | 4. | 2.10 | 100 | 0 |
| | 1.30 | 39 | 44 | | 1.70 | 81 | 5 |
| | | | | | 1.19 | 57 | 11 |
| 3. | 2.90 | 100 | 0 | | 0.88 | 42 | 23 |
| | 2.20 | 76 | 11 | | 0.80 | 38 | 38 |
| | 1.94 | 67 | 29 | | | | |
| | 1.70 | 59 | 46 | 5. | 2.39 | 100 | 0 |
| | | | | | 1.76 | 74 | 4 |
| 4. | 2.87 | 100 | 0 | | 1.19 | 50 | 11 |
| | 2.55 | 89 | 4 | | 0.95 | 40 | 16 |
| | 2.06 | 72 | 11 | | 0.73 | 31 | 29 |
| | 1.67 | 58 | 20 | | 0.69 | 29 | 42 |
| | 1.20 | 42 | 32 | | | | |
| | 1.05 | 37 | 35 | 6. | 2.64 | 100 | 0 |
| | | | | | 1.42 | 54 | 12 |
| 5. | 2.32 | 100 | 0 | | 1.34 | 51 | 20 |
| | 1.85 | 80 | 20 | | 1.15 | 44 | 26 |
| | 1.74 | 75 | 33 | | 0.90 | 34 | 39 |
| | 1.56 | 67 | 50 | | | | |
| | | | | 7. | 2.42 | 100 | 0 |
| | | | | | 2.00 | 83 | 5 |
| | | | | | 1.45 | 60 | 12 |
| | | | | | 1.20 | 50 | 20 |
| | | | | | 1.00 | 41 | 26 |
| | | | | | 0.89 | 37 | 40 |

The mean per cent heights of contraction were 90.5, 81.2, 66.9, 58.9, and 46.9 at 3, 6, 12, 20, and 34 seconds, respectively.

*For key, see Table 2.

Table 6

Response to HMC of muscles contracting in modified Tyrode's solution with no potassium

| D* | H | % | S | D | H | % | S |
|---|---|---|---|---|---|---|---|
| 2/4/64 | 2.73 | 100 | 0 | 9. | 3.57 | 100 | 0 |
| 2. | 2.00 | 73 | 7 | | 3.00 | 84 | 10 |
| | 1.40 | 51 | 17 | | 2.90 | 81 | 25 |
| | 1.23 | 45 | 41 | | 2.60 | 73 | 46 |
| 3. | 4.37 | 100 | 0 | 10. | 3.20 | 100 | 0 |
| | 2.00 | 46 | 10 | | 2.30 | 72 | 10 |
| | 1.52 | 35 | 29 | | 2.10 | 66 | 23 |
| | 1.29 | 30 | 40 | | 1.88 | 59 | 32 |
| 4. | 3.42 | 100 | 0 | 11. | 3.10 | 100 | 0 |
| | 2.38 | 70 | 12 | | 2.66 | 86 | 5 |
| | 2.00 | 58 | 27 | | 2.43 | 78 | 20 |
| | 1.90 | 56 | 41 | | 2.26 | 73 | 35 |
| 6. | 3.00 | 100 | 0 | 12. | 2.90 | 100 | 0 |
| | 2.61 | 87 | 8 | | 2.46 | 85 | 5 |
| | 2.30 | 77 | 22 | | 2.24 | 77 | 15 |
| | 2.12 | 71 | 37 | | 2.19 | 76 | 25 |
| | | | | | 2.06 | 71 | 48 |
| 7. | 2.70 | 100 | 0 | | | | |
| | 2.45 | 91 | 4 | 13. | 2.85 | 100 | 0 |
| | 2.35 | 87 | 22 | | 2.42 | 85 | 5 |
| | 2.28 | 84 | 37 | | 2.30 | 81 | 15 |
| | | | | | 2.03 | 71 | 30 |
| 8. | 3.73 | 100 | 0 | | 2.08 | 73 | 48 |
| | 3.00 | 81 | 4 | | | | |
| | 2.65 | 71 | 13 | 14. | 2.55 | 100 | 0 |
| | 2.36 | 63 | 30 | | 2.10 | 82 | 6 |
| | 2.13 | 57 | 41 | | 2.00 | 78 | 16 |
| | | | | | 1.90 | 75 | 25 |
| | | | | | 1.81 | 71 | 33 |
| | | | | | 1.72 | 67 | 46 |

The mean per cent heights of contraction were 90.6, 83.1, 75.0, 70.5, and 65.0 at 3, 6, 12, 20, and 34 seconds, respectively.

*For key, see Table 2.

Table 7

Response to HMC of muscles contracting in modified Tyrode's solution (0.228 grams calcium chloride per liter)

| D* | H | % | S | D | H | % | S |
|---|---|---|---|---|---|---|---|
| 2/20/64$_{I}$ | 2.41 | 100 | 0 | 2/20/64$_{II}$ | 2.48 | 100 | 0 |
| 8. | 1.27 | 53 | 6 | 2. | 1.66 | 67 | 6 |
| | 1.13 | 47 | 11 | | 1.16 | 47 | 10 |
| | 1.00 | 41 | 17 | | 1.00 | 40 | 17 |
| | 0.88 | 36 | 23 | | 0.90 | 36 | 23 |
| | 0.78 | 32 | 37 | | 0.68 | 27 | 30 |
| | | | | | 0.50 | 20 | 34 |
| 9. | 2.20 | 100 | 0 | | | | |
| | 1.31 | 60 | 6 | 3. | 3.48 | 100 | 0 |
| | 1.13 | 51 | 14 | | 2.76 | 79 | 4 |
| | 1.00 | 45 | 20 | | 1.66 | 48 | 10 |
| | 1.00 | 45 | 26 | | 1.32 | 38 | 16 |
| | 0.87 | 40 | 35 | | 0.92 | 26 | 23 |
| | | | | | 0.80 | 23 | 35 |
| 10. | 2.27 | 100 | 0 | | | | |
| | 1.98 | 87 | 5 | 4. | 2.85 | 100 | 0 |
| | 1.10 | 49 | 11 | | 1.73 | 61 | 7 |
| | 0.92 | 40 | 17 | | 1.30 | 46 | 13 |
| | 0.92 | 40 | 30 | | 0.91 | 32 | 19 |
| | 0.81 | 36 | 43 | | 0.80 | 28 | 26 |
| | | | | | 0.65 | 23 | 37 |
| 11. | 1.95 | 100 | 0 | | | | |
| | 1.10 | 56 | 6 | 5. | 3.13 | 100 | 0 |
| | 0.97 | 50 | 13 | | 2.15 | 69 | 6 |
| | 0.90 | 46 | 24 | | 1.17 | 37 | 12 |
| | 0.82 | 42 | 36 | | 0.90 | 29 | 19 |
| | | | | | 0.74 | 24 | 30 |
| 2/20/64$_{II}$ | 2.08 | 100 | 0 | | 0.71 | 23 | 37 |
| 1. | 1.70 | 82 | 5 | | | | |
| | 1.08 | 52 | 8 | 6. | 2.80 | 100 | 0 |
| | 0.64 | 31 | 13 | | 1.67 | 60 | 8 |
| | 0.52 | 25 | 19 | | 0.90 | 32 | 13 |
| | 0.34 | 16 | 28 | | 0.87 | 31 | 19 |
| | 0.32 | 15 | 35 | | 0.63 | 23 | 29 |
| | | | | | 0.60 | 21 | 36 |

The mean per cent heights of contraction were 82.5, 65.3, 44.3, 35.4, and 28.1 at 3, 6, 12, 20, and 34 seconds, respectively.

*For key, see Table 2.

Table 8

Response to HMC of muscles contracting in modified Tyrode's solution (0.185 grams calcium chloride per liter)

| D* | H | % | S | D | H | % | S |
|---|---|---|---|---|---|---|---|
| 2/20/64 II | 2.80 | 100 | 0 | 12. | 3.60 | 100 | 0 |
| 7. | 1.00 | 36 | 6 | | 2.45 | 68 | 6 |
| | 0.70 | 25 | 15 | | 1.80 | 50 | 12 |
| | 0.58 | 21 | 22 | | 1.38 | 38 | 19 |
| | 0.70 | 25 | 30 | | 1.35 | 37 | 30 |
| | 0.60 | 21 | 40 | | 0.90 | 25 | 33 |
| | | | | | 0.80 | 22 | 39 |
| 8. | 2.86 | 100 | 0 | | | | |
| | 2.00 | 70 | 5 | 13. | 2.88 | 100 | 0 |
| | 1.24 | 43 | 9 | | 1.63 | 57 | 4 |
| | 1.20 | 42 | 17 | | 1.40 | 49 | 11 |
| | 0.90 | 31 | 21 | | 1.29 | 45 | 18 |
| | 0.70 | 24 | 28 | | 0.90 | 31 | 24 |
| | 0.50 | 17 | 37 | | 0.75 | 26 | 31 |
| | | | | | 0.50 | 17 | 38 |
| 9. | 2.90 | 100 | 0 | | | | |
| | 0.94 | 32 | 6 | 14. | 3.07 | 100 | 0 |
| | 0.80 | 28 | 13 | | 2.80 | 91 | 10 |
| | 0.66 | 23 | 26 | | 2.25 | 73 | 14 |
| | 0.70 | 24 | 32 | | 1.82 | 59 | 19 |
| | 0.66 | 23 | 39 | | 1.49 | 49 | 23 |
| | | | | | 1.30 | 42 | 29 |
| 10. | 4.40 | 100 | 0 | | 0.97 | 32 | 39 |
| | 3.63 | 82 | 6 | | | | |
| | 3.50 | 80 | 13 | 15. | 2.90 | 100 | 0 |
| | 2.90 | 66 | 18 | | 2.45 | 84 | 6 |
| | 1.62 | 37 | 22 | | 1.57 | 54 | 10 |
| | 1.23 | 28 | 28 | | 1.48 | 51 | 16 |
| | 1.10 | 25 | 41 | | 1.39 | 48 | 22 |
| | | | | | 1.10 | 38 | 34 |
| 11. | 2.84 | 100 | 0 | | | | |
| | 1.19 | 42 | 5 | 16. | 2.42 | 100 | 0 |
| | 0.97 | 34 | 12 | | 1.60 | 66 | 5 |
| | 0.80 | 28 | 24 | | 1.20 | 50 | 11 |
| | 0.60 | 21 | 29 | | 0.80 | 33 | 15 |
| | 0.50 | 18 | 33 | | 0.54 | 22 | 27 |
| | 0.45 | 16 | 40 | | 0.40 | 17 | 31 |
| | | | | | 0.35 | 15 | 50 |

The mean per cent heights of contraction were 78.0, 61.3, 48.9, 37.3, and 24.8 at 3, 6, 12, 20, and 34 seconds, respectively.

*For key, see Table 2.

Table 9

Response to HMC of muscles contracting in modified Tyrode's solution (0.153 grams calcium chloride per liter)

| D* | H | % | S | D | H | % | S |
|---|---|---|---|---|---|---|---|
| 2/20/64$_{II}$ | 2.60 | 100 | 0 | 2. | 3.15 | 100 | 0 |
| 17. | 1.56 | 60 | 11 | | 2.95 | 94 | 6 |
| | 1.24 | 48 | 17 | | 1.55 | 49 | 14 |
| | 0.93 | 36 | 24 | | 1.30 | 41 | 21 |
| | 0.70 | 27 | 29 | | 0.82 | 26 | 27 |
| | 0.57 | 22 | 44 | | 0.50 | 16 | 33 |
| | | | | | 0.40 | 13 | 41 |
| 18. | 2.65 | 100 | 0 | | | | |
| | 2.45 | 92 | 7 | 3. | 3.00 | 100 | 0 |
| | 1.73 | 65 | 13 | | 1.95 | 65 | 3 |
| | 1.55 | 58 | 23 | | 1.06 | 35 | 14 |
| | 1.00 | 38 | 32 | | 0.40 | 13 | 18 |
| | 0.60 | 23 | 40 | | 0.38 | 13 | 28 |
| | | | | | 0.22 | 7 | 33 |
| 19. | 2.58 | 100 | 0 | | | | |
| | 1.66 | 64 | 7 | 4. | 3.84 | 100 | 0 |
| | 1.20 | 47 | 18 | | 2.70 | 70 | 6 |
| | 0.72 | 28 | 28 | | 2.00 | 52 | 12 |
| | 0.65 | 25 | 37 | | 1.55 | 40 | 17 |
| | | | | | 1.44 | 38 | 22 |
| 20. | 1.98 | 100 | 0 | | 1.00 | 26 | 30 |
| | 1.08 | 55 | 10 | | 0.98 | 25 | 36 |
| | 0.90 | 45 | 18 | | | | |
| | 0.65 | 33 | 26 | 5. | 3.60 | 100 | 0 |
| | 0.47 | 24 | 37 | | 2.10 | 58 | 7 |
| | | | | | 1.12 | 31 | 18 |
| 2/20/64$_{III}$ | 2.65 | 100 | 0 | | 0.64 | 18 | 28 |
| 1. | 2.10 | 79 | 10 | | 0.65 | 18 | 35 |
| | 1.84 | 69 | 17 | | | | |
| | 1.41 | 53 | 24 | | | | |
| | 1.00 | 38 | 29 | | | | |
| | 0.80 | 30 | 36 | | | | |

The mean per cent heights of contraction were 86.1, 75.8, 56.2, 41.2 and 24.2 at 3, 6, 12, 20, and 34 seconds, respectively.

*For key, see Table 2.

Table 10

Response to HMC of muscles contracting in modified Tyrode's solution (0.098 grams calcium chloride per liter)

| D* | H | % | S | D. | H | % | S |
|---|---|---|---|---|---|---|---|
| 2/20/64 III | 2.33 | 100 | 0 | 10. | 4.25 | 100 | 0 |
| 6. | 1.87 | 80 | 5 | | 3.30 | 78 | 5 |
| | 1.26 | 54 | 13 | | 2.45 | 58 | 11 |
| | 0.80 | 34 | 21 | | 2.16 | 51 | 22 |
| | 0.35 | 15 | 26 | | 2.00 | 47 | 28 |
| | 0.35 | 15 | 33 | | 1.85 | 44 | 42 |
| | 0.30 | 13 | 39 | | | | |
| | | | | 11. | 4.40 | 100 | 0 |
| 7. | 4.55 | 100 | 0 | | 4.10 | 93 | 6 |
| | 3.72 | 80 | 6 | | 2.95 | 67 | 12 |
| | 3.50 | 75 | 14 | | 2.60 | 59 | 28 |
| | 2.64 | 57 | 21 | | 2.50 | 57 | 37 |
| | 2.37 | 51 | 26 | | | | |
| | 2.10 | 45 | 32 | 12. | 4.10 | 100 | 0 |
| | 1.62 | 35 | 44 | | 3.80 | 93 | 8 |
| | | | | | 3.45 | 84 | 14 |
| 8. | 4.55 | 100 | 0 | | 2.47 | 60 | 24 |
| | 3.40 | 75 | 10 | | 2.40 | 59 | 37 |
| | 2.80 | 62 | 19 | | | | |
| | 2.70 | 59 | 23 | 13. | 4.00 | 100 | 0 |
| | 2.70 | 55 | 30 | | 3.75 | 94 | 5 |
| | 2.50 | 48 | 36 | | 2.95 | 74 | 11 |
| | | | | | 2.50 | 63 | 23 |
| 9. | 4.18 | 100 | 0 | | 2.40 | 60 | 30 |
| | 3.78 | 90 | 9 | | 2.15 | 54 | 43 |
| | 2.90 | 69 | 15 | | | | |
| | 2.78 | 66 | 24 | | | | |
| | 2.70 | 65 | 30 | | | | |
| | 2.50 | 60 | 38 | | | | |

The mean per cent heights of contraction were 91.9, 84.6, 71.1, 59.1, and 48.0 at 3, 6, 12, 20, and 34 seconds, respectively.

*For key, see Table 2.

Table 11

Response to HMC of muscles contracting in modified Tyrode's solution with no calcium

| D* | H | % | S | D | H | % | S |
|---|---|---|---|---|---|---|---|
| 2/20/64$_{III}$ 14. | 3.08 | 100 | 0 | 2/20/64$_{IV}$ 3. | 3.05 | 100 | 0 |
| | 1.95 | 63 | 6 | | 2.70 | 88 | 7 |
| | 2.52 | 82 | 14 | | 2.37 | 78 | 20 |
| | 2.46 | 80 | 19 | | 2.30 | 75 | 28 |
| | 1.70 | 55 | 34 | | 2.28 | 74 | 41 |
| 15. | 2.65 | 100 | 0 | 4. | 4.30 | 100 | 0 |
| | 2.48 | 94 | 5 | | 3.49 | 81 | 5 |
| | 2.65 | 100 | 12 | | 2.65 | 61 | 17 |
| | 2.55 | 96 | 28 | | 2.40 | 56 | 30 |
| | 2.00 | 76 | 39 | | 2.28 | 53 | 45 |
| 16. | 3.10 | 100 | 0 | 5. | 3.85 | 100 | 0 |
| | 2.86 | 96 | 4 | | 3.40 | 88 | 10 |
| | 2.24 | 72 | 16 | | 2.36 | 61 | 22 |
| | 2.43 | 78 | 27 | | 1.86 | 48 | 40 |
| | 2.00 | 64 | 41 | | | | |
| | | | | 6. | 3.68 | 100 | 0 |
| 2/20/64$_{IV}$ 1. | 1.55 | 100 | 0 | | 3.40 | 92 | 4 |
| | 1.10 | 71 | 7 | | 2.65 | 72 | 19 |
| | 1.07 | 69 | 16 | | 1.90 | 52 | 31 |
| | 0.97 | 63 | 26 | | 1.85 | 50 | 43 |
| | 0.84 | 54 | 38 | | | | |
| 2. | 3.50 | 100 | 0 | | | | |
| | 3.10 | 89 | 8 | | | | |
| | 2.90 | 83 | 17 | | | | |
| | 2.70 | 77 | 36 | | | | |

The mean per cent heights of contraction were 92.4, 85.0, 81.2, 74.6, and 63.4 at 3, 6, 12, 20, and 34 seconds, respectively.

*For key, see Table 2.

Table 12

Milliliters of titrant needed to develop permanent turbidity--comparison of HMC with sodium versenate

| Trial 1, sodium oxalate added | | | |
|---|---|---|---|
| Solution Number | Water Controls | Solution with HMC | Solution with Sodium Versenate |
| 1 | 0.60 | 1.16 | 12.30 |
| 2 | 0.40 | 0.81 | 12.50 |
| 3 | 0.33 | 1.00 | --- |
| Average | 0.44 | 0.99 | 12.40 |

| Trial 2, calcium chloride added | | | |
|---|---|---|---|
| Solution Number | Water Controls | Solution with HMC | Solution with Sodium Versenate |
| 1 | 4.63 | 4.84 | 6.35 |
| 2 | 4.47 | 4.71 | 6.60 |
| 3 | 5.20 | 5.04 | 5.86 |
| Average | 4.77 | 4.86 | 6.27 |